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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

We have investigated the mechanisms by which estrogen serves as a survival factor for breast cancer, during chemotherapy or irradiation treatment in-vitro. UV irradiation for one minute or four hour treatment with taxol results in substantial apoptosis of breast cancer cell lines. This is due to a c-jun N-terminal kinase activation, leading to an inactivating phosphorylation of Bcl_2 and Bcl_{x-L} proteins, leading to caspase 9 activation and cell death. Estrogen blocks this proximally, at JNK activation. Independently, estrogen stimulates ERK MAP kinase activity which also contributes to cell survival. This was published in Molecular Endocrinology 14(9):1434-47, 2000.

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Table of Contents

Introduction

Estrogen promotes an increased incidence of breast cancer in women, while anti-estrogen therapy both limits recurrences and prevents the development of primary disease in genetically predisposed individuals. The purpose of the studies proposed in our grant is to determine the cellular mechanisms by which estrogen enhances the survival of breast cancer.

Body

In conjunction with the approved "Statement of Work", we first established that UV irradiation for one minute, or taxol treatment for four hours resulted in significant increases of breast cancer cell death. This was dependent upon the stimulation of c-Jun N-terminal kinase (JNK) activity by these two treatments, and was shown in two breast cancer cell lines that expressed estrogen receptors, MCF-7 and ZR-75-1 cells. The necessity of JNK participation was shown in that transfecting the cells to express a dominant-negative JNK construct substantially the apoptotic effects of UV or taxol. We further found that estradiol (E_2) or E_2-BSA, a cell impermeable E_2 that only binds plasma membrane estrogen receptors (ER) each significantly blocked JNK activation in this setting. The JNK-activating effects of UV or taxol led to the inactivating phosphorylation of BcL_2 and $BcL_{x\text{-}L}$, which are important survival proteins in the breast cancer cells. This was again blocked by either E_2 or E_2-BSA. The inhibition of BcL_2 or $BcL_{x\text{-}L}$ phosphorylation by the sex steroids was reversed upon transfecting /expressing a mildly constituitively active JNK-1 protein. The inactivation of BcL_2 and $BcL_{x\text{-}L}$ function by UV or taxol led to the cleavage and activation of caspase-9, the initiator of the death effector cascade, in a JNK-dependent fashion. Again, E_2 or E_2-BSA substantially blocked these events, and ultimately rescued 62% of the cells from apoptosis, and 67% of this resulted from blocking the JNK-induced pathway. We then investigated additional signaling from the plasma membrane ER, by which E_2 or E_2-BSA promoted cell survival. We found that the steroids induced the activation of ERK MAP kinase. Blockage of ERK with the MEK (MAP kinase kinase) soluble inhibitor, PD 98059, prevented 33% of the anti-apoptotic effects of the steroids, in the setting of UV or taxol exposure to the cells. Thus, these two signaling pathways that derive from estrogen action at the plasma membrane account for 100% of the breast cancer cell survival effects of estrogen, at least during a six-hour period. These results suggest that a target to therapeutically prevent these important actions of estrogen would be to antagonize their effects, specifically at the membrane receptor. These results were published in *Molecular Endocrinology* 14:1434047, 2000.

The next set of studies in our approved Statement of Work, was to investigate the actions of E_2 on vascular endothelial cells. This is potentially referable as to how E_2 could serve as an angiogenesis factor, for the tumor vasculature, to promote breast cancer propagation. These data are now published in *Journal of Biological Chemistry* 275:38540-46, 2000. Briefly, we found that E_2 activated a novel signaling pathway from the cell surface estrogen receptor. This resulted in the activation of the MAP kinase $P^{38}\beta$, the linked activation of MAPKAP/-2 kinase, and the phosphorylation of heat shock protein 27. This pathway was essential to the ability of E_2 to prevent actin cytoskeleton de-arrangement, promote endothelial cell (EC) survival after hypoxia insult, and to promote EC migration and new blood vessel formation (angiogenesis). These data allow mechanistic understanding of E_2 action at this important vascular cell, and again point to the potential therapeutic target of the plasma membrane ER.

Key Research Accomplishments

- Identification of mechanisms by which estradiol leads to breast cancer cell survival and prevents the functions of radiation or chemotherapy to kill breast cancer cells.

- Proof that the plasma membrane estrogen receptor importantly contributes to the actions of the sex steroid in breast cancer.

- Determination of a novel and important signaling pathway from the plasma membrane ER, that results in the survival and stimulation of new blood vessel formation. This is potentially important to understand estrogen effects on the tumor vasculature, and identifies a therapeutic target.

Reportable Outcomes

Abstracts and presentations

1. Razandi M, Pedram A, Levin ER. Estrogen preserves endothelial cell form and function through membrane receptor activation of p38-MAPKAP-HSP27 pathway. Presented at the 82nd Annual Meeting of the Endocrine Society, Toronto, Canada, June 2000.
2. Razandi M, Pedram A, Levin ER. Estrogen signals to anti-apoptosis in breast cancer through the membrane. Presented at the 82nd Annual Meeting of the Endocrine Society, Toronto, Canada, June 2000.

Manuscripts

1. Razandi M, Pedram A, Levin ER. Plasma membrane estrogen receptors signal to anti-apoptosis in breast cancer. Molecular Endocrinology 14(9):1434-47, 2000.
2. Razandi M, Pedram A, Levin ER. Estrogen signals to preservation of endothelial cell form and function. Journal of Biological Chemistry 275(49):38540-46, 2000.

Conclusions

Our findings provide a mechanistic understanding of the cellular events by which estrogen contributes to the survival and development of breast cancer. Further, our findings provide detailed support to the therapeutic recommendation that women should not receive estrogen hormones when treated for breast cancer, and should not be given estrogen after the disease has been diagnosed. Furthermore, little information is available on the cellular mechanisms by which estrogen could theoretically affect the tumor vasculature. We show here potential signaling pathways from a cell surface estrogen receptor that are important in this regard. This may change our therapeutic or preventive intervention strategies, to target new antagonists or SERMS that have cell membrane ER action as well as nuclear receptor activity.

References

None.

Appendices

Two articles attached.

Plasma Membrane Estrogen Receptors Signal to Antiapoptosis in Breast Cancer

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Chemotherapy or irradiation treatment induces breast cancer cell apoptosis, but this can be limited by estradiol (E_2) through unknown mechanisms. To investigate this, we subjected estrogen receptor-expressing human breast cancer cells (MCF-7 and ZR-75–1) to paclitaxel (taxol) or to UV irradiation. Marked increases in cell apoptosis were induced, but these were significantly reversed by incubation with E_2. Taxol or UV stimulated c-Jun N-terminal kinase (JNK) activity, which was inhibited by E_2. Expression of a dominant-negative Jnk-1 protein strongly prevented taxol- or UV-induced apoptosis, whereas E_2 inhibition of apoptosis was reversed by expression of constitutively active Jnk-1. As targets for participation in apoptosis, Bcl-2 and Bcl-xl were phosphorylated in response to JNK activation by taxol or UV; this was prevented by E_2. Taxol or UV activated caspase activity in a JNK-dependent fashion and caused the cleavage of procaspase-9 to caspase-9, each inhibited by E_2. Independently, the steroid also activated extracellular signal-regulated protein kinase activity, which contributed to the anti-apoptotic effects. We report novel and rapid mechanisms by which E_2 prevents chemotherapy or radiation-induced apoptosis of breast cancer, probably mediated through the plasma membrane estrogen receptor. (Molecular Endocrinology 14: 1434–1447, 2000)

INTRODUCTION

Estrogen receptors are expressed in approximately 65% of human breast cancer, implying that this sex steroid plays an important role in the development and propagation of the disease (1, 2). Approximately one third of women with breast cancer respond to ablative endocrine therapy (3, 4), and anti-estrogens positively influence the course of established breast cancer (5) or prevent the development of primary disease (6). Based upon *in vitro* and *in vivo* data, estrogen probably acts as both a growth factor and a survival factor for breast cancer (1, 2).

The ability of estradiol (E_2) to act as a survival factor for breast cancer is not well understood, but a substantial part of the effects probably occur through the prevention of programmed cell death, apoptosis (7). Apoptosis is often initiated when a cell is exposed to a stressful stimulus, which then triggers a transmembrane signal to an intracellular protease cascade, primarily composed of the caspase family (8). As a result, intracellular enzymes are activated that cleave DNA and cause cell shrinkage, chromatin condensation, and membrane blebbing. In the early course of establishment of breast cancer, a cytokine response could induce apoptosis of the cancer cells via the activation of cell surface receptors for tumor necrosis factor, as an example (9). In established breast cancer, treatment with chemotherapy or irradiation induces apoptosis. *In vitro*, breast cancer treatment with chemotherapy is markedly less effective in the setting of estrogen (10, 11). Thus, E_2 may establish a survival advantage in this setting, but the mechanisms of this effect are not well understood.

The actions of E_2 are traditionally thought to be mediated by the nuclear estrogen receptor (ER), through the regulation of target gene transcription (12). This occurs when ER either binds estrogen response elements on the promoters of target genes, or acts through protein-protein interactions involving a variety of coactivators, corepressors, and the basal transcriptional machinery protein complex. Emerging evidence, however, has implicated a second distinct mechanism of E_2 action, where this steroid binds a putative plasma membrane ER and enacts signal transduction (13, 14). Each mechanism could work cooperatively or distinctly to effect cell biological actions. Conceivably, the ability of E_2 to prevent apoptosis in several target cells (15, 16) could be initiated through signal trans-

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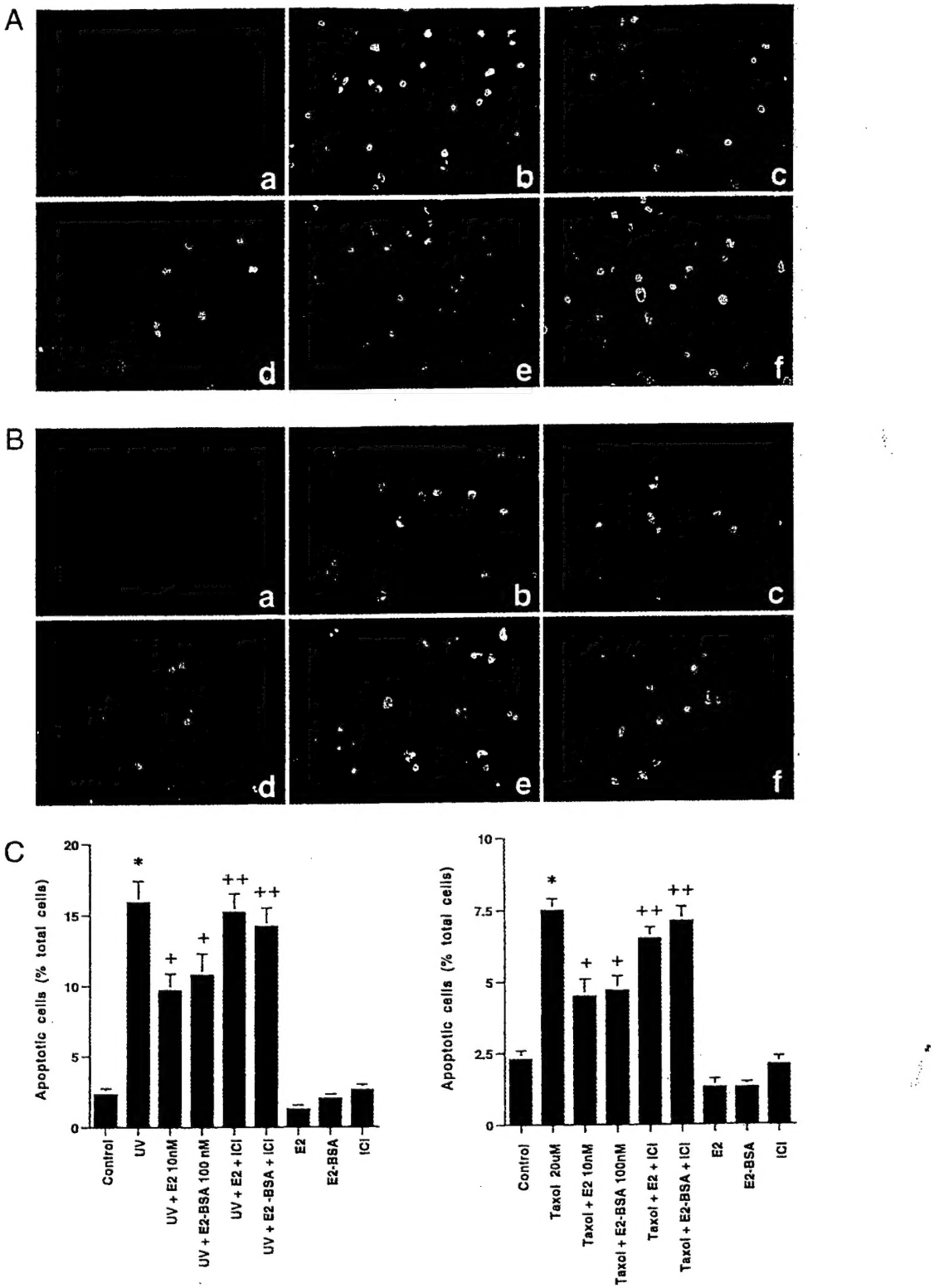

Fig. 1. A, Apoptosis of MCF-7 Cells Is Induced by UV Irradiation Exposure for 1 Min (b), as Demonstrated by Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Staining Compared with Control Cells (a)

This is inhibited by 10 nM E_2 (c) or 100 nM E_2-BSA (d), which is reversed by 1 μM ICI182,780, an ER antagonist (e and f, respectively). B, Taxol (20 μM) exposure for 4 h induces apoptosis in MCF-7 cells (b), inhibited by E_2 or E_2-BSA (c and d), again reversed by ICI182,780 (e and f). The study shown here was repeated three times. *Bar graphs* show the mean ± SEM number of apoptotic cells in each condition, based on combined data, which are shown *below* the composites (*C*). Apoptotic cells are stained *yellow/green* compared with viable cells (*red*) stained with propidium iodide.

duction mediated through the plasma membrane receptor.

As the anti-apoptotic effects of E_2 are poorly understood, we investigated the mechanism of action and whether the membrane receptor mediates these effects of the sex steroid.

RESULTS

Apoptosis is Inhibited by Membrane ER Ligation

MCF-7 and ZR-75–1 cells were subjected either to a brief (1-min) UV irradiation, followed by 4-h incubation, or to 20 μM taxol treatment for 4 h. As shown in Fig. 1A, UV (panel b) induced 13% of the MCF-7 breast cancer cells to undergo apoptosis, compared with 1% in the control cells (panel a). Preincubation with 10 nM E_2 or 100 nM E_2-BSA (panels c and d), significantly prevented the effect of UV, lowering cell death to 6% and 7%, respectively. The protective effects of either estrogen compound were reversed by ICI182,780, the specific ER antagonist (panels e and f). Similar effects were seen for taxol-induced apoptosis (Fig. 1B), although taxol was not as potent in this regard as UV exposure. The data are summated in the bar graphs below each composite figure. These short exposures were chosen to support the idea that a rapid, nongenomic effect of E_2 might be involved. In contrast, neither 100 nM testosterone nor progesterone had any significant effect on UV or taxol-induced apoptosis in MCF-7 cells (data not shown).

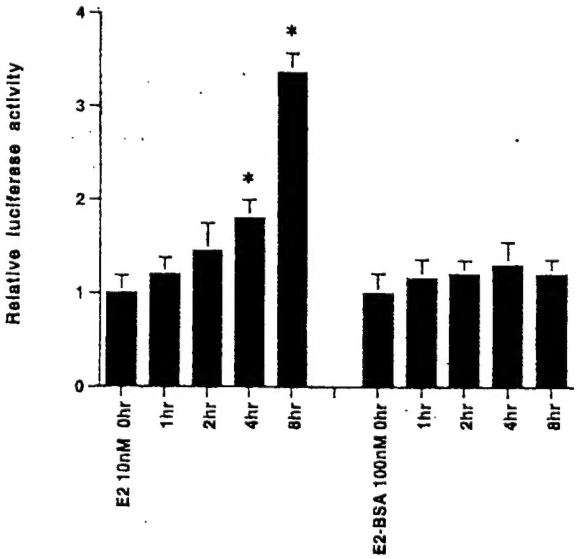

Fig. 2. Activation of an ERE-Luciferase Reporter Over Time by E_2 (10 nM), but Not by E_2-BSA (100 nM)

The results are the mean ± SEM luciferase activity, determined from triplicate determinations per time point in each experiment. All data were combined from three separate experiments. *, $P < 0.05$, by ANOVA and Scheffe's test for time zero *vs.* 4 or 6 h.

Table 1. Apoptosis in Breast Cancer Cells after Exposure to Ultraviolet (UV) Irradiation or Taxol, in the Presence or Absence of Estrogen

Treatment	% Apoptosis (BrdU incorporation)	
	MCF-7 cells	ZR-75-1 cells
Control	1.8 ± 0.5	0.8 ± 0.2
UV	13.0 ± 1.0[a]	12.1 ± 0.9[a]
UV + E2 10 nM	4.1 ± 0.5[b]	3.0 ± 0.3[b]
UV + E2 + ICI 1 μM	10.1 ± 0.8[a]	9.3 ± 0.7[a]
UV + E2-BSA 100 nM	5.9 ± 0.9[b]	—
UV + E2-BSA + ICI 1 μM	11.3 ± 1.2[a]	—
Control	1.8 ± 0.5	0.8 ± 0.2
Taxol 20 μM	7.6 ± 0.8[a]	6.5 ± 0.5[a]
Taxol + E2 10 nM	3.4 ± 0.4[b]	2.7 ± 0.3[b]
Taxol + E2 + ICI 1 μM	6.6 ± 0.5[a]	5.3 ± 0.5[a]
Taxol + E2-BSA 100 nM	4.1 ± 0.3[b]	—
Taxol + E2-BSA + ICI 1 μM	6.1 ± 0.4[a]	—

Apoptosis was determined by FACS analysis of cells stained with propidium iodide and BrdU. Data are the mean ± SEM from three experiments combined.

[a] $P < 0.05$ by ANOVA plus Scheffe's for control *vs.* condition or UV or taxol versus UV or taxol + ICI.

[b] $P < 0.05$ for UV or taxol *vs.* UV or taxol + E_2. ICI alone was not different from control (data not shown).

These results indicate that E_2 is rapidly acting through a specific plasma membrane ER to inhibit apoptosis. This is supported by the similar effects of E_2-BSA, a compound that has been shown by several laboratories to neither enter the cell nor bind/activate the nuclear ER (17–19). E_2-BSA has previously been shown to be less potent than E_2, perhaps due to the steric hindrance of E_2 accessing its receptor, when conjugated to BSA (17–19). We have previously shown that BSA by itself does not affect signaling (18).

To further support the idea that E_2 and E_2-BSA are acting through a membrane ER, we transiently transfected the cells with an estrogen response element (ERE)-luciferase reporter construct, as previously described (19). Over 8 h, E_2 (10 nM) significantly stimulated the reporter activity, but E_2-BSA did not (Fig. 2). This indicates that E_2-BSA does not enter the cell to bind the nuclear ER by 8 h, determined at several times later than the inhibition of apoptosis shown here (4 h). These results also indicate that E_2 does not substantially dissociate from the BSA, although this has recently been called into question (see *Discussion*).

We also assessed apoptosis by labeling MCF-7 and another ER-positive breast cancer cell, ZR-75–1, with bromodeoxyuridine and propidium iodide; we then determined cell death by fluorescent-activated cell sorting (FACS) analysis. As shown in Table 1, UV and taxol induced at least, 6- and 4-fold respective increases in programmed death in either cell line. E_2 afforded between a 66–80% protection against apoptosis across both conditions and both cell lines. The effects of E_2-BSA were comparable to those of E_2, and all ste-

Table 2. Annexin-V Binding to the Membrane of Breast Cancer Cells Exposed to Ultraviolet (UV) Irradiation or Taxol, with or without Estrogen

Treatment	% Apoptosis (Annexin V binding)	
	MCF7 cells	ZR-75-1 cells
Control	0.8 ± 0.2	2.2 ± 0.5
UV	12.8 ± 2.1[a]	18.7 ± 2.1[a]
UV + E2 10 nM	2.5 ± 0.3[b]	10.1 ± 1.2[b]
UV + E2 + ICI 1 μM	9.7 ± 1.2[a]	15.5 ± 1.7[a]
UV + E2-BSA 100 nM	5.7 ± 0.6[b]	—
UV + E2-BSA + ICI 1 μM	9.7 ± 1.3[a]	—
Control	0.8 ± 0.2	2.2 ± 0.5
Taxol 20 μM	8.3 ± 1.2[a]	11.7 ± 2.1[a]
Taxol + E2 10 nM	2.7 ± 0.5[b]	6.1 ± 1.1[b]
Taxol + E2 + ICI 1 μM	5.6 ± 0.3[a]	9.1 ± 1.3[a]
Taxol + E2-BSA 100 nM	2.2 ± 0.6[b]	—
Taxol + E2-BSA + ICI 1 μM	5.4 ± 0.6[a]	—

Apoptosis was determined by FACS analysis of cells binding Annexin-V-FITC to phosphatidylserine on the membrane. Data are the mean ± SEM from three experiments combined.
[a] $P < 0.05$ by ANOVA plus Scheffe's for control *vs.* condition, or UV or taxol *vs.* same + ICI.
[b] $P < 0.05$ for UV or taxol *vs.* UV or taxol + E_2. ICI was not different from control (data not shown).

roid actions were reversed 70–90% by ICI182,780. In contrast, E_2 had no effect on UV- or taxol-induced apoptosis in an ER-negative cell line, HCC1569 (data not shown).

To examine an early event in apoptosis, the cells were assessed for cell membrane binding of annexin V, again determined by FACS. In the cell undergoing programmed cell death, annexin V can bind phosphatidlyserine, which is expressed in the outer plasma membrane leaflet of dying cells. In both ER-positive breast cancer cell lines, UV or taxol induced 16-fold (MCF-7) and 8.5-fold (ZR-75) increases in annexin binding compared with control cells. E_2 or E_2-BSA significantly reversed this by 52–86% across all conditions and both cell lines (Table 2). ICI182,780 substantially reversed the effects of either E_2 or E_2-BSA.

Apoptosis Is Modulated through c-Jun N-terminal kinase (JNK) Activity

We then determined whether the induction of apoptosis by UV or taxol was dependent on JNK activity and could be modified by E_2. JNK is known to mediate the activation of apoptosis in several cell types, in response to various stressors (20). We first found that either UV or taxol could significantly activate JNK activity by 7- and 2.5-fold, respectively, in MCF-7, and significantly in ZR-75–1 cells (Fig. 3A, *upper* and *lower panels*). We also found that either E_2 or E_2-BSA caused a 50–67% reduction in the stimulated JNK activity seen in response to either stressor in both cell lines. The comparable effects of the two estrogen compounds were blocked by ICI182,780, and when considered along with the rapidity of inhibition by E_2 (determined after 15 min of cell exposure), these results support a membrane ER as mediating this action. The effects of E_2 or E_2-BSA were also concentration related, with significant inhibition seen at 1 and 10 nm E_2 in both cell lines (Fig. 3B). In contrast, E_2 did not reduce UV- or taxol stimulated-JNK activity in HCC-1569 cells (data not shown).

A critical issue is whether JNK activation is necessary and sufficient for the induction of apoptosis in this setting. As shown in Fig. 4A, MCF-7 cells transfected to express a dominant-negative Jnk-1 showed a significant reduction in the degree of apoptosis induced by UV [panel c *vs.* a (control)] or by taxol [panel d *vs.* b (control)]. Control MCF-7 cells were transfected with the empty plasmid vector and subjected to either of the two apoptotic stimuli. The reversal induced by dominant-negative Jnk-1 was substantial (63–67% for UV or taxol; Fig. 4B), which must be considered in the context that transfection efficiency does not allow complete inhibition of JNK activation. We had previously determined our transfection efficiency using this construct as approximately 75% (21). These results show the requirement of JNK for apoptosis induced by these two agents. Because UV- or taxol-induced JNK activation and apoptosis were blocked by this steroid, we believe that this is a novel target for the anti-apoptotic effects of estrogen.

To further support this contention, we transfected MCF-7 cells with a mildly constituitively active Jnk-1 expression plasmid, a vector that we and others have previously characterized (20, 21). We then exposed the cells to UV or taxol in the presence or absence of E_2 or E_2-BSA. We found that active Jnk-1 resulted in a nearly complete reversal of the ability of E_2 or E_2-BSA to block UV- or taxol-induced apoptosis (Fig. 4C).

Bcl-2 and Bcl-xl Serve as Substrates for Jnk

What JNK targets are critical for the apoptosis-inducing action of this enzyme? It has recently been shown that the anti-apoptotic protein Bcl-2 can be phosphorylated. In several cell types; phosphorylation by, for instance, protein kinase A or c-Jun kinase (JNK), down-regulates Bcl-2 actions to prevent cell death (22–24), although concomitant activation of phosphatases or phosphorylation of a different site may lead to activation of the protein in some contexts (25). However, recent data clearly indicate that JNK can inactivate Bcl-2 function by directly phosphorylating the loop domain of this protein (24). We determined that UV or taxol was capable of significantly stimulating the phosphorylation of Bcl-2 (Fig. 5A). Further, the phosphorylation was mainly attributable to JNK activation, as transfection of the MCF-7 cells with dominant-negative Jnk-1 almost completely reversed this effect of UV or taxol.

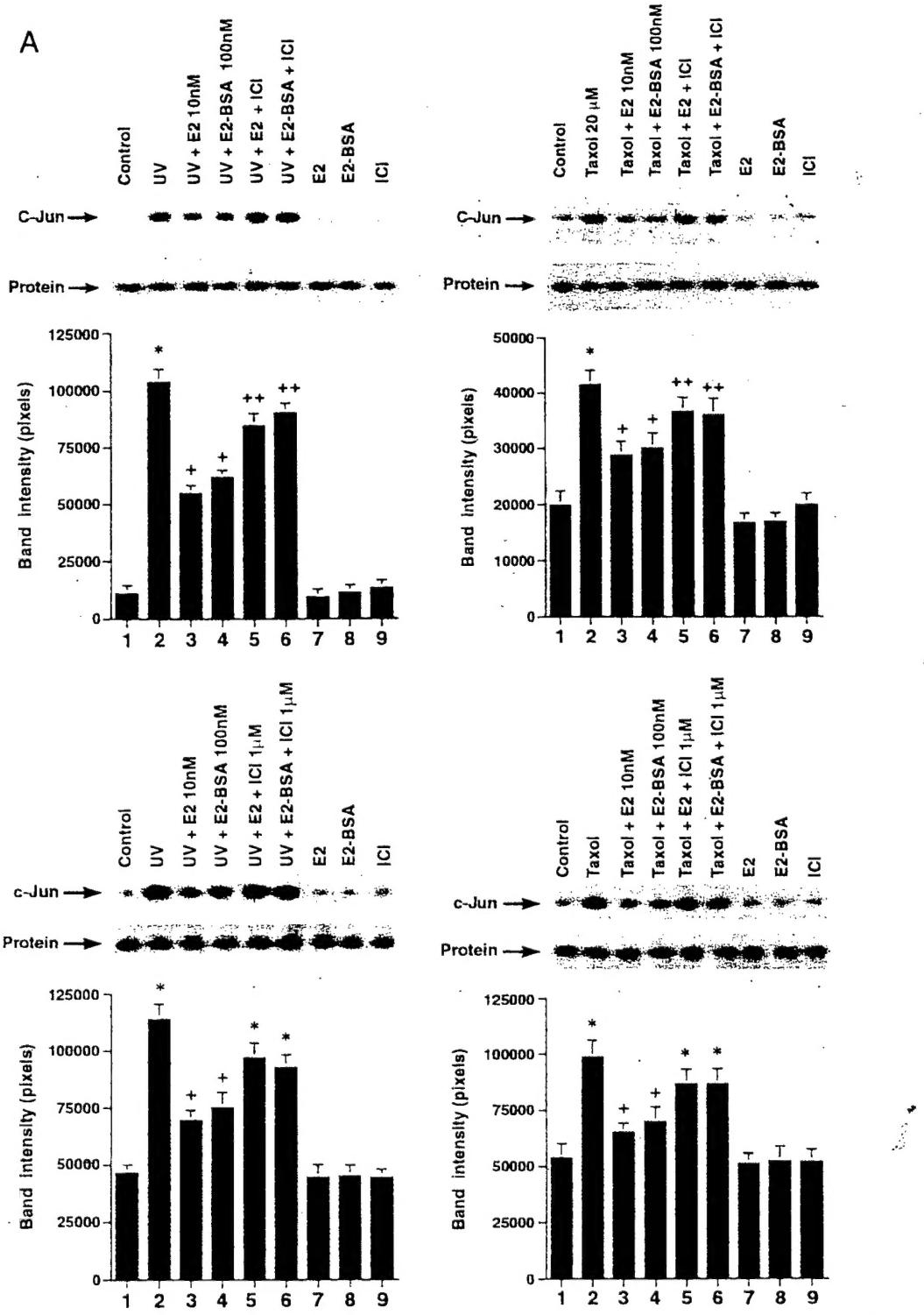

Fig. 3. A, The c-Jun Kinase Activity in MCF-7 Cells (*upper*) and ZR-75–1 Cells (*lower*) after 15-Min Exposure to UV (*left*) or Taxol (*right*), in the Presence or Absence of E_2, E_2-BSA, or ICI182,780

JNK was immunoprecipitated from the treated MCF-7 cells, as described in *Materials and Methods*. A representative experiment of JNK activity directed against GST-c-Jun-(1–79) as substrate protein is shown, with the Jnk-1 immunoblots *below* each condition. The *bar graph* represents mean results ± SE of three experiments combined. *, $P < 0.05$ for control *vs.* UV or taxol; +, $P < 0.05$ for UV or taxol *vs.* UV or taxol plus E_2 or E_2-BSA; ++, $P < 0.05$ for UV or taxol plus E_2 or E_2-BSA *vs.* the former plus ICI. B, Concentration-related inhibition of UV- or taxol-stimulated JNK activity by E_2 or E_2-BSA in MCF-7 (*left*) and ZR-75–1 (*right*). The data reflect three separate experiments combined.

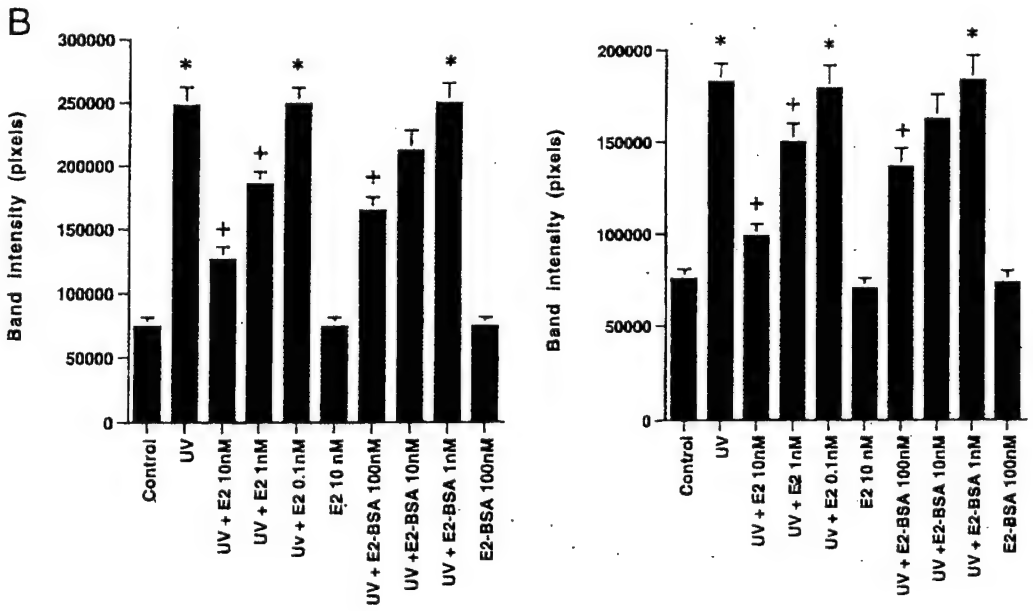

Fig. 3. Continued.

Another important anti-apoptotic protein in this family is Bcl-xl. We similarly found that UV and taxol induced JNK-dependent phosphorylation of this protein in MCF-7 cells (Fig. 5B). However, in this situation, another kinase may additionally contribute. As E_2 or E_2-BSA inhibits JNK activation, we hypothesized that they would block UV- or taxol-induced phosphorylation of Bcl-2 and Bcl-xl. We found this to be the case, in that either estrogen compound significantly inhibited the phosphorylation of these two proteins by 53–64% (Fig. 5C). To support a JNK-related mechanism of action, the blocking of either Bcl-2 or Bcl-xl phosphorylation by E_2 was substantially reversed when a constituitively active Jnk-1 was expressed in the MCF-7 cells (Fig. 5C, lanes 8 and 9). This identifies a novel downstream target for the anti-apoptotic effects of the membrane ER, and the mechanism is probably mediated through the inhibition of JNK activation.

Caspase Activation Is JNK Dependent and Is Inhibited by E_2 or E_2-BSA

The inactivation of Bcl-2 or Bcl-xl induced by UV- or taxol-induced phosphorylation might lead to activation of caspase activity (22, 23). We determined this in whole cell lysates using a fluorogenic substrate that assesses caspase-4, -5, and -9 activities (26). UV- or taxol-treated cells showed 91% and 75% increases, respectively, in caspase activity, compared with nontreated, control MCF-7 cells (Table 3). Adding E_2-BSA or E_2 reduced the enhanced caspase activity by 41%–53% across both conditions. To determine a role for JNK, MCF-7 cells were transfected with the empty plasmid, pcDNA3, or the dominant negative Jnk-1 construct. In MCF-7 cells transfected with pcDNA3, the apoptosis was higher than that in nontransfected cells. However, UV and taxol still significantly enhanced caspase activity in this setting, by 68% and 51%, respectively (Table 3). Expression of Flag-Jnk-1 APF significantly reduced this stimulated caspase activity by 65% and 51%, respectively.

Active caspase-9 facilitates the cleavage and activation of the death effector caspases, resulting in DNA fragmentation and apoptosis. Caspase-9 activation mainly results from proteolytic processing of procaspase-9. This processing is indirectly restrained by Bcl-2 or Bcl-xl and is directly activated through association with the cytochrome *c*-Apaf-1 complex. We therefore determined whether UV and taxol cleaved procaspase-9 to caspase-9, yielding detectable active fragments of the zymogen. In control MCF-7 cells, only the 46-kDa zymogen was detected. Both taxol and UV induced cleavage of the procaspase, yielding smaller molecular mass bands at 34 kDa, which were detected after stressor exposure (Fig. 6). E_2 or E_2-BSA substantially prevented the cleavage of procaspase-9 in the setting of either stressor for the MCF-7 cells.

Extracellular Signal-Regulated Protein Kinase (ERK) Activation Contributes to Antiapoptosis

Might there be a contribution of other signaling molecules to the protective actions of E_2? We determined the possible role of the ERK member of the mitogen-activated protein kinase family. Both E_2 and E_2-BSA stimulated ERK activity by about 3-fold after 10-min exposure to the MCF-7 cells, and this was prevented

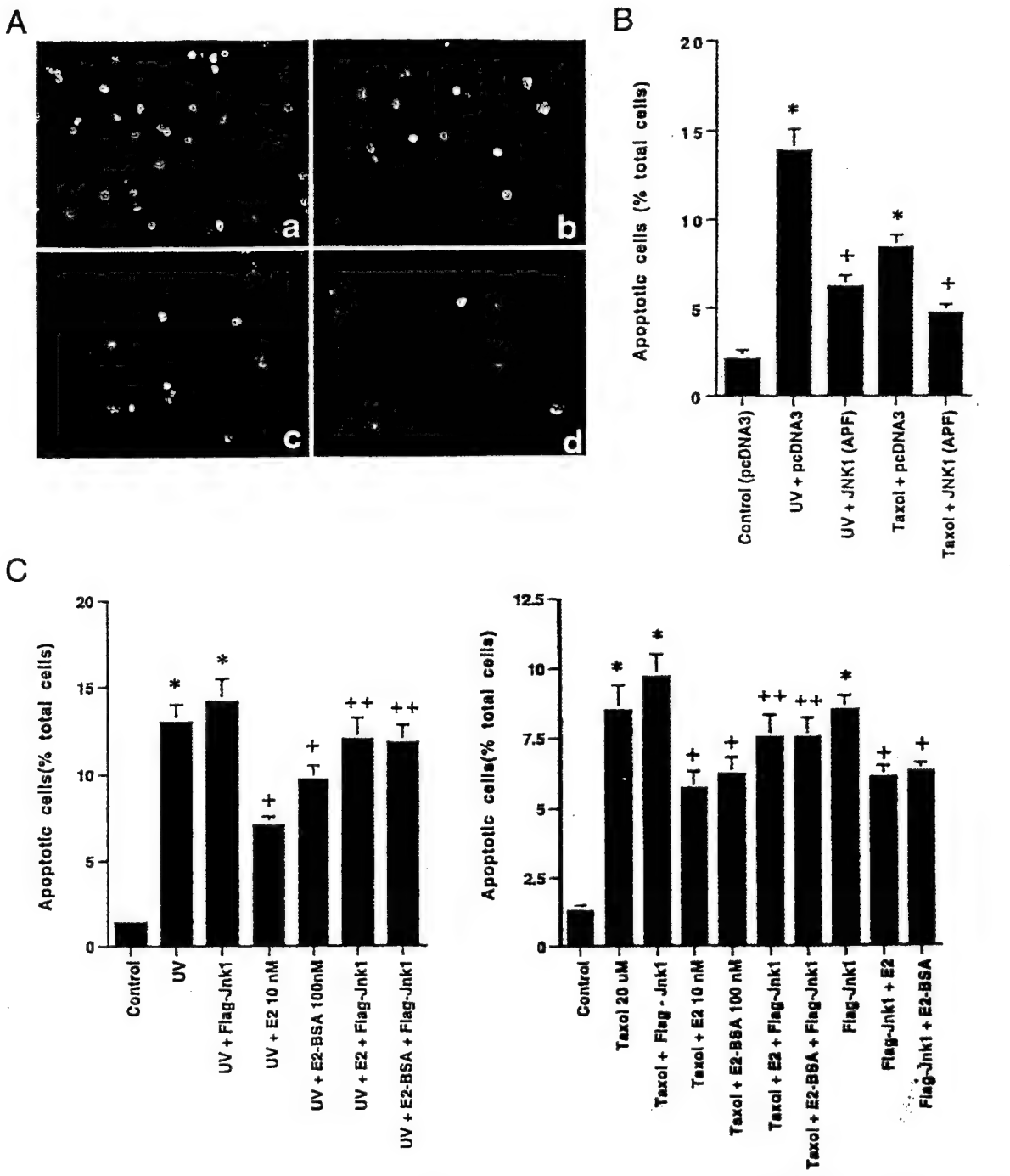

Fig. 4. A, Apoptosis of pcDNA3-Transfected MCF-7 Cells in Response to UV (a) or Taxol (b) Is Substantially Greater Than That in Cells Transfected with Dominant-Negative Jnk-1 (pcDNA3 Flag-Jnk-1 APF), Then Exposed to UV (c) or Taxol (d)

MCF-7 cells were transfected with dominant-negative (dom-neg) Jnk-1, recovered, then subjected to UV or taxol. B, Quantitation of three apoptosis experiments is shown in the *bar graph*. Control apoptosis (pcDNA3 transfected, but not subjected to UV or taxol) was 2% and is not shown. *, $P < 0.05$ for UV or taxol *vs.* UV or taxol plus dom-neg Jnk-1. C, Expression of constituitively active Jnk-1 (Flag-Jnk-1) reverses the E_2 or E_2-BSA inhibition of UV (*left*)- or taxol (*right*)-induced apoptosis. Data are from three experiments combined. *, $P < 0.05$ for UV or taxol *vs.* UV or taxol plus E_2 or E_2-BSA; +, $P < 0.05$ for UV or taxol plus E_2 or E_2-BSA *vs.* the former in the presence of active Jnk-1

by ICI182,780 (Fig. 7A). In the setting of UV or taxol, incubation of MCF-7 cells with PD 98059, a mitogen-activated extracellular protein kinase kinase (MEK) inhibitor, partially reversed the anti-apoptotic effects of E_2 or E_2-BSA; this reversal ranged from 33–48% (Fig. 7B). These results indicate that ERK activation by E_2

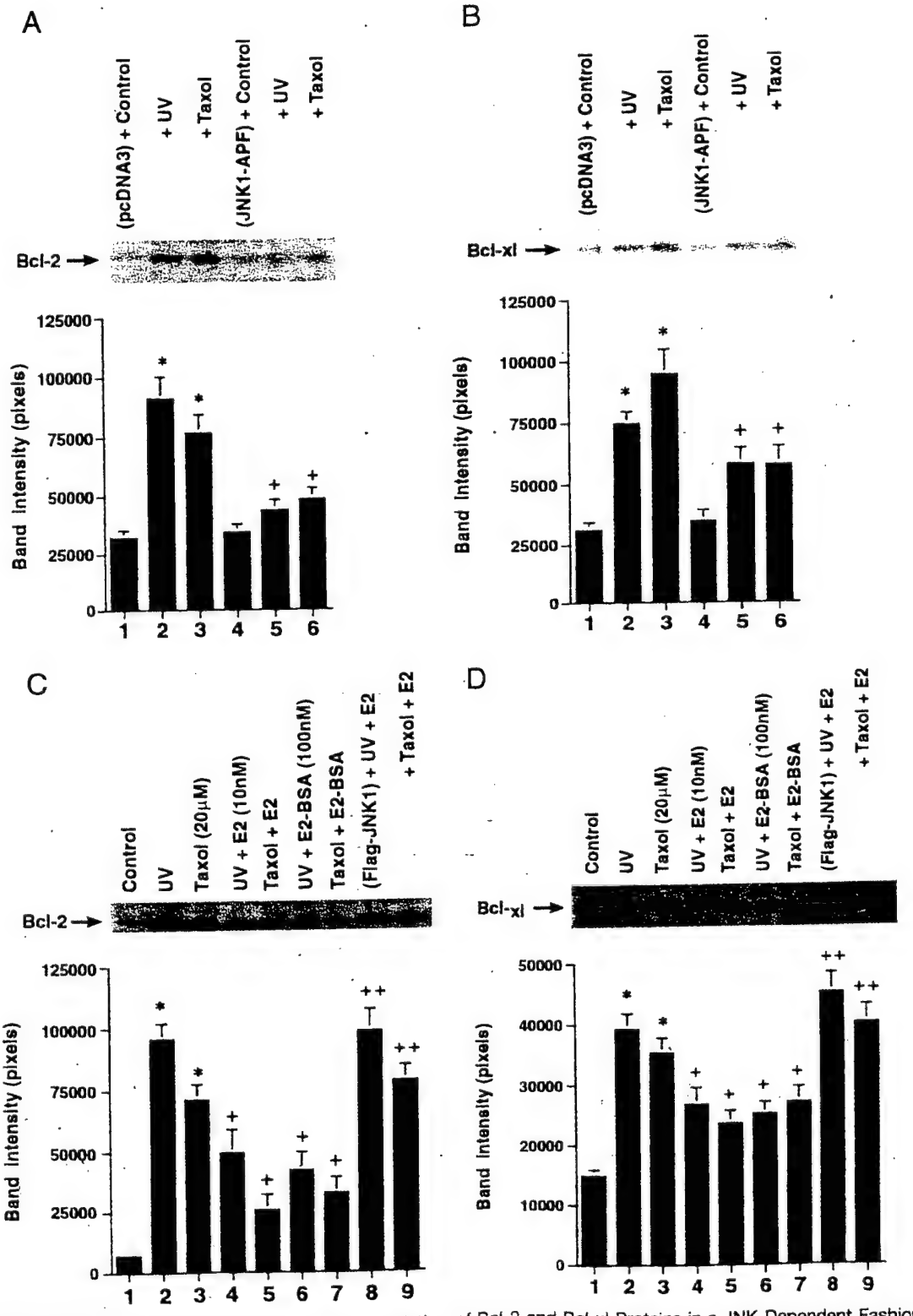

Fig. 5. A, UV or Taxol Induces the Increased Phosphorylation of Bcl-2 and Bcl-xl Proteins in a JNK-Dependent Fashion after 15-Min Incubation

JNK1-APF, Dominant-negative (dom-neg) Jnk-1. A representative study is shown, repeated three times to constitute the bar graph. *, $P < 0.05$ for control *vs.* UV or taxol; +, $P < 0.05$ for UV or taxol *vs.* UV or taxol plus dom-neg Jnk-1. B, UV or taxol induces the increased phosphorylation of Bcl-xl protein in a JNK-dependent manner. A representative study from three experiments is shown. C, E_2 or E_2-BSA inhibits UV- or taxol-induced phosphorylation of Bcl-2 (*left*) and Bcl-xl (*right*), which is reversed by expressing active Jnk-1 (Flag-JNK-1).

Table 3. E2 inhibits UV or Taxol-Induced Activation of Caspase Activity

Condition	Absorbance units/ (mg protein)
Control	203 ± 11
UV	387 ± 14[a]
UV + E2 10 nM	299 ± 16[b]
UV + E2-BSA 100 nM	308 ± 12[b]
Taxol 20 μM	355 ± 18[a]
Taxol + E_2	275 ± 13[b]
Taxol + E_2-BSA	291 ± 11[b]
E_2	208 ± 14
E_2-BSA	198 ± 12
pcDNA3	301 ± 21
pcDNA3 + UV	504 ± 24[a]
pcDNA3 + Taxol	452 ± 18[a]
pcDNA3 Flag-JNK-1 APF + UV	372 ± 18[c]
pcDNA3 Flag-JNK-1 APF + Taxol	379 ± 20[c]
pcDNA3 Flag-JNK-1 APF	278 ± 19

Data are the mean ± SEM from three experiments combined. [a] $P < 0.05$ for control *vs.* condition by ANOVA plus Schefe's test; [b] $P < 0.05$ for condition *vs.* condition plus E_2 or E_2-BSA; [c] $P < 0.05$ for condition *vs.* condition plus dominant negative c-jun-1 kinase (pcDNA3 Flag-JNK-1 APF). Caspase activity was determined as described in *Materials and Methods* using a fluorogenic substrate that identifies caspase 4, 5 and 9 activities.

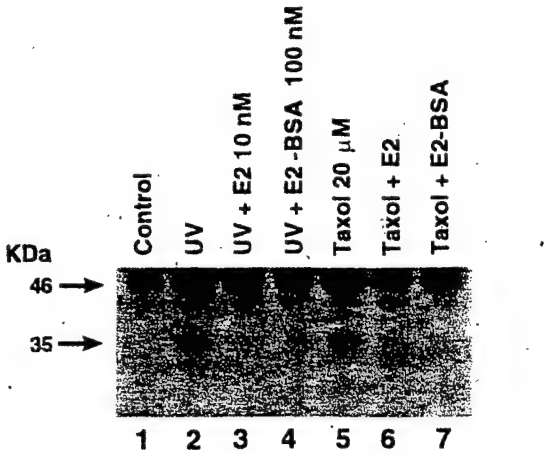

Fig. 6. Procaspase-9 Is Cleaved by UV (1-Min Exposure) or Taxol (20 μM), Which Is Prevented by E_2 or E_2-BSA

MCF-7 cells were exposed to UV or taxol in the presence or absence of E_2 or E_2-BSA, as described, then incubated for a total of 4 h. Cell lysates were then immunoprecipitated for caspase-9, using a monoclonal antibody that recognizes unprocessed and processed forms of the caspase-9 zymogen. After SDS-PAGE separation and membrane transfer, Western blot for caspase-9 was carried out. A representative study is shown, repeated three times.

occurs through the plasma membrane ER, and that this contributes to the anti-apoptotic effects of the sex steroid.

DISCUSSION

E_2 is an acknowledged growth and/or survival factor for several cell types (1–3, 27, 28). Prenatally, E_2 could limit the programmed cell death mechanism of remodeling, which is used as part of developmental plasticity in target organs (29). Postnatally, E_2 protection of the ovarian follicle ensures fertility (30), whereas antiapoptosis in vascular cells probably contributes to the lowered incidence of arterial disease in estrogen-replaced postmenopausal women (31). However, the effect of E_2 is cell and context specific. When advantageous, E_2 can also induce apoptosis to protect against bone resorption by inducing the death of osteoclasts (32). A very important effect of E_2 in this regard is the unfortunate ability of this sex steroid to prevent breast cancer cells from undergoing apoptosis in response to chemotherapy or radiation.

Here, we have shown that E_2 prevents UV- or taxol-induced apoptosis of ER-positive breast cancer cell lines and have uncovered some novel mechanisms of action. E_2 partially, but significantly, prevents UV- or taxol-induced JNK activation and separately stimulates the activation of ERK. Each mechanism contributes to the anti-apoptotic effects of this steroid (Fig. 8). E_2 acts through a putative plasma membrane ER (13, 14), a receptor that has not yet been physically isolated, but for which strong functional evidence has now emerged (19, 33–35). We have recently shown that a single cDNA for ERα (or ERβ) can result in the expression of both nuclear and plasma membrane binding proteins. The membrane receptor is a G protein-linked receptor capable of signaling through multiple pathways after G protein activation (19). In the studies reported here, we show that E_2 or E_2-BSA inhibits the UV- or taxol-induced rapid activation of JNK. These effects are reversed by an ER antagonist.

Several laboratories have previously shown that E_2-BSA does not activate the nuclear ER (17, 19), and that this compound can be used as a membrane ER-specific ligand. However, this has recently been called into question. Stevis *et al.* proposed that commercially prepared E_2-BSA substantially deconjugates to free E_2 and BSA, and that the conjugated E_2-BSA, but not free E_2, has nonspecific effects to activate signal transduction, at least in neural cancer cells (36). They also did not find nuclear ER activation by intact E_2-BSA. In the studies presented here, E_2 and E_2-BSA act similarly to inhibit signaling to apoptosis, and their effects are always significantly reversed by an ER antagonist. Additionally, E_2, but not E_2-BSA, activated an ERE-luciferase reporter gene; this indicates that E_2-BSA did not bind the nuclear ER and did not dissociate substantially into free E_2 and BSA. Furthermore, the focus should be not whether E_2-BSA is a useful reagent to activate the membrane ER, but, rather, what are the nongenomic, rapid actions of E_2? Importantly, the signal effects of E_2 rapidly occur (within 5–15 min) and are unlikely to represent an unprecedented action of a nuclear receptor.

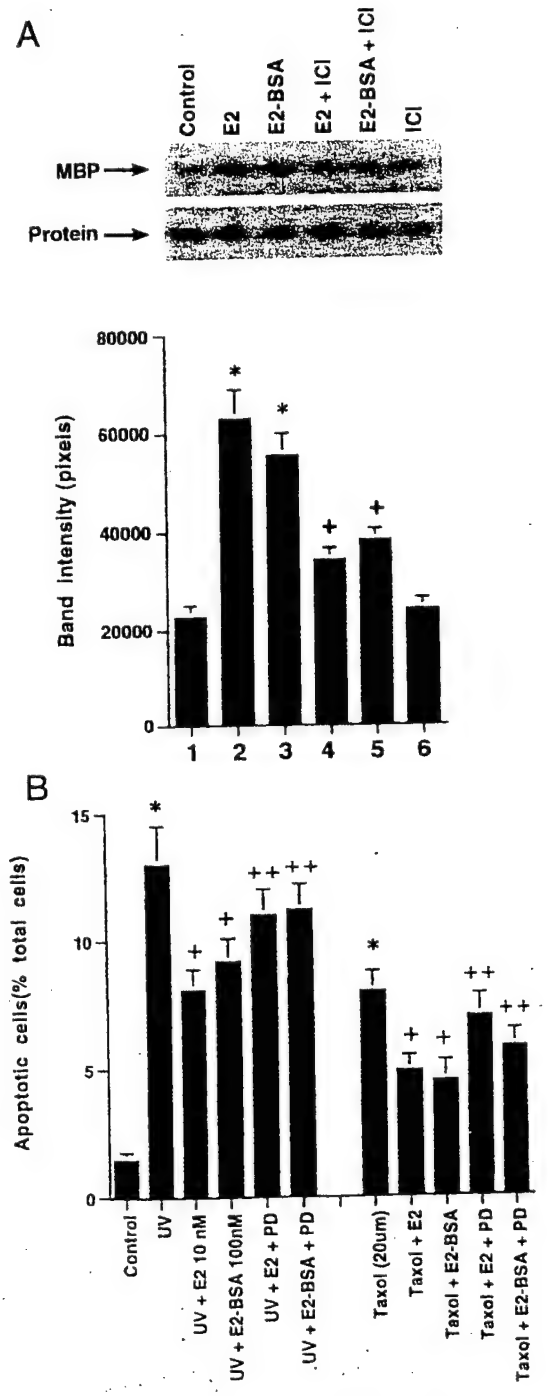

Fig. 7. A, E_2 and E_2-BSA Stimulate ERK Activity

MCF-7 cells were incubated with the steroids for 10 min, ERK activity was immunoprecipitated from cell lysate, and activity was determined against myelin basic protein (MBP). A representative study is shown, repeated two additional times. B, *Bar graph* of three experiments combined delineating the effects of the MEK inhibitor, PD 98059, to partially reverse the ability of E_2 or E_2-BSA to prevent apoptosis. Data are the mean ± SE from three experiments. *, $P < 0.05$ for control *vs.* UV or taxol; +, $P < 0.05$ is UV or taxol *vs.* either treatment plus E_2 or E_2-BSA; ++, $P < 0.05$ is UV or taxol plus E_2 or E_2-BSA *vs.* the former plus PD 98059.

The nongenomic actions of E_2 probably result from ERα ligation by the steroid. The latter conclusion derives from the fact that MCF-7 cells express almost exclusively the ERα receptor (37). Furthermore, we have previously shown in CHO cells transfected to singly express either ER that E_2 ligation of ERα inhibits basal JNK activity (and stimulates ERK activation), whereas in CHO-ERβ cells, E_2 activates JNK (22). It has been established here and previously that JNK is essential to apoptosis induced by UV or taxol in several cell types (24, 38, 39).

We have further defined downstream targets for E_2-related antiapoptosis in breast cancer. UV and taxol were found to stimulate the phosphorylation, and hence the inactivation, of Bcl-2. These agents also stimulated phosphorylation of the Bcl-xl protein in MCF-7 cells. This occurs mainly through a JNK-mediated mechanism, which is also inhibited through the membrane ER. Recently, taxol-induced JNK activation has been shown to directly phosphorylate/inactivate Bcl-2 (24). Active Bcl-2 (and Bcl-xl) is proposed to prevent cytochrome *c* release from mitochondria (40), and therefore inhibit the complexing of Apaf-1, cytochrome *c*, and procaspase-9. In the presence of ATP, cytochrome *c* induces the oligomerization of Apaf-1, which then cleaves the procaspase-9 zymogen, yielding active caspase-9. We report here that taxol or UV induces the activation of caspase activity in a JNK-dependent fashion and also induces the cleavage of the procaspase-9 zymogen to caspase-9. These events are significantly prevented by E_2 or E_2-BSA.

Caspase-9 cleaves/activates the death effector caspases, such as procaspase-3 or -7, thereby effecting apoptosis (41, 42). Caspase-3 is not functional in MCF-7 cells (43), but other effector caspases, such as caspase-7, are present (confirmed by us). In addition to its actions in preventing the release of cytochrome *c*, Bcl-xl has been shown to form a ternary complex with Apaf-1 and caspase-9, perhaps inhibiting the

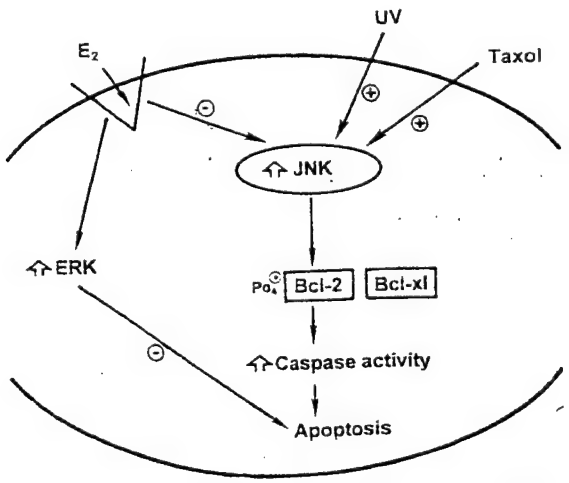

Fig. 8. Schema of E_2 Acting through the Plasma Membrane ER to Signal to Antiapoptosis

ability of Apaf-1 to activate caspase-9 (44). Active Bcl-2 has many important functions in preventing cell death (40, 45), and each, theoretically, could be affected by and contribute to the effects of E_2 shown here. It has previously been demonstrated that E_2 can stimulate the transcription and protein synthesis of Bcl-2 (11, 46). Thus, E_2 can prevent cell death by acute and more chronic modulation of both the activity and levels of this anti-apoptotic protein. This may be an example of coordinated cellular actions between the membrane and nuclear ERs, respectively.

The novel finding that UV and taxol induce Bcl-xl phosphorylation through a JNK-dependent mechanism deserves comment. This important anti-apoptotic protein can form homo- or heterodimers with other family members, including Bcl-2, Bad, Bax, *etc.*, and it is believed that these interactions underlie the effects of Bcl-xl. However, it is not clear what the effect of phosphorylation is on Bcl-xl action. Analogous to Bcl-2, Bcl-xl contains a 60-amino acid loop domain that partially suppresses the anti-apoptotic functions of this protein (47). The identical domain in Bcl-2 can be phosphorylated by JNK, and this phosphorylation disables Bcl-2 cell survival function (24, 48). We would predict that this sequence is a target for Bcl-xl phosphorylation via JNK, causing inactivation of anti-apoptotic function. Very recently, Kufe and colleagues demonstrated that ionizing radiation causes the translocation of JNK to the mitochondria of leukemia cells, where JNK phosphorylates Bcl-xl (49). Expression of a phosphorylation mutant Bcl-xl in these cells prevents ionizing radiation-induced apoptosis, perhaps because the mutant protein cannot be inactivated by phosphorylation. Collectively, the data suggest that the ability of E_2 to inhibit the JNK-induced, inactivating phosphorylation of Bcl-2 provides a partial understanding of the anti-apoptotic effects of this sex steroid and probably extends to Bcl-xl function as well.

It is likely that E_2 stimulates other signal transduction mechanisms to inhibit apoptosis. In MCF-7, E_2 stimulates the cascade that activates the ERK member of the mitogen-activated protein kinase family, and importantly contributes to DNA synthesis in this cell (50, 51). This kinase is recognized to mediate cell proliferation in response to a variety of growth factors targeting a myriad of cell types (52, 53) and has also been proposed to act as a survival protein (54, 55). We show here that E_2 or E_2-BSA activates ERK, and that inhibition of activated ERK with the soluble and specific MEK-1 inhibitor, PD 98059, partially reverses E_2-induced antiapoptosis in MCF-7 cells. This indicates that ERK contributes to E_2 effects in this regard. Singer *et al.* showed that excitotoxicity-induced necrosis of neurons can be prevented by E_2 or nerve growth factor, mediated through ERK signaling to unknown downstream targets (56). Several mechanisms of ERK-induced protection against antiapoptosis have recently been elucidated. These include the phosphorylation of the pro-apoptotic BAD protein at serine 112 (57) and the activation of cAMP response element-binding protein-mediated transcription (58). Thus, it is likely that several pathways that originate from the plasma membrane ER lead to the preservation of target cells, and that it is the overall balance of pro- and anti-apoptotic signals that determines the fate of a cell.

Exactly where JNK (or ERK) fits in the apoptotic pathway is not clear, but we and others (48) have shown that JNK is upstream of Bcl-2. The complete effector pathways that prevent apoptosis will need to be defined in a situation-specific context (59). Furthermore, taxol is a microtubule-stabilizing agent, which theoretically acts through several mechanisms to induce apoptosis (60, 61). These conceivably could also be influenced by E_2 in a JNK-independent fashion.

Based upon these studies, we speculate that the actions of the membrane ER in breast cancer could underlie the ability of E_2 to effect cell survival *in vivo* (62). Understanding the mechanisms by which E_2 induces antiapoptosis in cancer provides theoretical targets to prevent this undesirable action. Membrane ER-specific antagonists that are targeted to cancer cells might be a future means to enhance the response to therapy. This might also allow women to take hormone replacement to preserve desirable genomic effects of the nuclear ER. To support this strategy, further understanding of the discrete actions of E_2 at both the cell membrane and the nucleus must occur.

MATERIALS AND METHODS

Cells and Apoptosis Determination

MCF-7 cells were grown on 18-mm coverslips in 12-well culture dishes in DMEM/F-12 medium without phenol red, but with added charcoal-stripped serum. For apoptosis studies, the cells were subjected to 1 min of UV irradiation (20 J/cm^2) or paclitaxel (taxol; 20 μM) and incubated for 4 h at 37 C in the presence or absence of 10 nM E_2 or 100 nM E_2-BSA. At the end of incubation, the cells were washed with PBS and fixed with 1% freshly prepared paraformaldehyde in PBS, pH 7.4, at 4 C overnight. Apoptosis was then determined by the terminal deoxynucleotidyl transferase-stimulated incorporation of nucleotides into the 3-OH end of damaged DNA in the cell, detected by fluorescent antibodies to the nucleotides (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling), using a kit from Intergen (Purchase, NY). From each experimental condition, 400 cells were visually scored for apoptosis, viewed by fluorescence microscopy using standard fluorescein excitation and emission filters. In addition, FACS-based cell counting for apoptosis was carried out after bromodeoxyuridine labeling, for MCF-7 and another ER-positive cell line, ZR-75–1. Apoptosis in both of these cell lines was also determined by FACS detection of annexin V binding using a kit (Becton Dickinson and Co., Mountain View, CA). In early apoptosis, the plasma membrane phospholipid, phosphatidylserine, translocates from the inner to the outer membrane leaflet. In cells undergoing apoptosis, phosphatidylserine is then available to bind phospholipid-binding proteins, such as annexin V. HCC1569 cells served as a control, ER-negative breast cancer cell line for these experiments.

Transient Transfections

Breast cancer cell lines were grown to 60–70% confluence in DMEM/F-12 (MCF-7) medium or RPMI-1640 (ZR-75-1 and HCC1569), without phenol red but with 10% FBS. The cells were then washed and transiently transfected with 5–10 μg of fusion plasmids after optimization depending on the plate size and the amount of cells. The plasmids included c-Jun wild type or dominant-negative mutant (see below), ERE-simian virus 40 luciferase (provided by Dr. B. Gehm), or respective backbone vectors. DNA was amplified and isolated using the QIAGEN maxi-prep kit (Chatsworth, CA), and care was taken to minimize carryover of salts, alcohol, or other confounding reagents. Transfections were performed with Lipofectamine reagent (Life Technologies, Inc., Grand Island, NY); cells were incubated with liposome-DNA complexes at 37 C for approximately 5 h, followed by overnight recovery in DMEM-F-12 medium containing 10% FBS. Then, before experimental treatment, cells were synchronized in serum-free DMEM-F-12 for 24 h and treated with 17β-E_2 and/or related compounds. Cotransfections with a green fluorescent protein expression vector (Promega Corp., Madison, WI) indicated approximately 70–75% efficiency of transfection. Luciferase activity was determined as previously described (19, 63). The concentration of E_2-BSA was calculated from the number of E_2 molecules attached to each BSA molecule.

Kinase Assays

The c-Jun kinase activity was determined as previously described (21). Briefly, MCF-7 cells were incubated under various conditions for 15 min, then the cells were lysed, and lysate was immunoprecipitated for Jnk-1. JNK activity was determined against GST-c-Jun-(1–79) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as substrate. Laser densitometry of the phosphorylated bands was used for quantitation, and three experiments were combined for statistical analysis. Immunoblot of Jnk-1 protein assured equal amounts of protein loaded in each condition. In some experiments, wild-type (pcDNA3Flag-Jnk-1) or dominant-negative Jnk-1 (pcDNA3 Flag-Jnk-1 APF) (58) was transfected into MCF-7 cells as previously described (21, 58). ERK activity was determined as previously described (18). MCF-7 cells were synchronized in serum- and growth factor-free medium for 24 h, then incubated with E_2 or E_2-BSA for 10 min, and immunoprecipitated ERK activity from cell lysates was determined against myelin basic protein as the substrate. For apoptosis experiments, cells were subjected to UV or taxol treatment with or without E_2 or E_2-BSA in the absence or presence of the specific ERK kinase (MEK-1) inhibitor, PD 98059 (10 μM).

Bcl-2 and Bcl-xl Phosphorylation

MCF-7 cells were incubated for 1 h at 37 C in phosphate-free medium containing 5% dialyzed FBS. At the end of the incubation, cells were washed and labeled with ^{32}P (final concentration, 0.2 mCi/ml) for 2 h at 37 C in a CO_2 incubator. Cells were further incubated with or without taxol, UV, and E_2 or E_2-BSA for 1 h. At the end of the labeling period, cells were washed with ice-cold PBS, then lysed with buffer for 30 min on ice. The lysates were microcentrifuged, and Bcl-2 and Bcl-xl immunoprecipitation from the supernatant was conducted using specific antibodies (Santa Cruz Biotechnology, Inc.) at 4 C. After centrifugation and washing, the immunoprecipitated Bcl-2 and Bcl-xl were resolved by SDS-PAGE on a 12% gel, followed by autoradiography.

Caspase Activity and Zymogen Proteolysis

Cultured MCF-7 cells in 100-mm dishes were exposed to UV or taxol as described for the previous experiments. The cytosolic extracts were repeatedly frozen in extraction buffer and thawed as previously described (26). Cell lysates were then diluted and incubated with 1 μM fluorescent substrate (caspase-4, -5, and -9 substrate) for 30 min at 30 C. At the end of the incubation, the fluorescence of the cleaved substrates was determined using a spectrofluorometer, set at an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Cleavage of the zymogen, procaspase-9, was assessed. MCF-7 cells were cultured as described, then treated with UV (1 min) or taxol (20 μM) for 4 h in the absence or presence of E_2 or E_2-BSA. At the end of the 4-h incubation, the cells were washed, then lysed in buffer for 30 min on ice. The lysates were microfuged, and the supernatants were immunoprecipitated with anti-caspase-9 polyclonal antibody, raised against a peptide corresponding to the unique amino acids 299–318 of human caspase-9 (Cayman Chemical, Ann Arbor, MI). The immunoprecipitants were then analyzed by Western blotting using the ECL kit from Amersham Pharmacia Biotech (Arlington Heights, IL).

Statistical Analysis

Pooled data from multiple experiments were compared by ANOVA and Scheffe's test, using the StatView statistical program ($P < 0.05$ as significant). Bar graphs represent the mean ± SEM from at least three experiments.

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Estrogen Signals to the Preservation of Endothelial Cell Form and Function*

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Estrogen is important for the primary prevention of vascular disease in young women, but the mechanisms of protection at the vascular cell are still largely unknown. Although traditionally thought of as a nuclear transcription factor, the estrogen receptor has also been identified in the cell plasma membrane to signal but serve largely undefined roles. Here we show that estradiol (E2) rapidly activates p38β mitogen-activated protein kinase in endothelial cells (EC), which activates the mitogen-activated protein kinase-activated protein kinase-2 and the phosphorylation of heat shock protein 27. The sex steroid preserves the EC stress fiber formation and actin and membrane integrity in the setting of metabolic insult. E2 also prevents hypoxia-induced apoptosis and induces both the migration of EC and the formation of primitive capillary tubes. These effects are reversed by the inhibition of p38β, by the expression of a dominant-negative mitogen-activated protein kinase-activated protein kinase-2 protein, or by the expression of a phosphorylation site mutant heat shock protein 27. E2 signaling from the membrane helps preserve the EC structure and function, defining potentially important vascular-protective effects of this sex steroid.

E2[1] has been reported to serve a significant function in the vasculature to prevent the *primary* development of cardiovascular disease in women (1). This occurs through multiple potential and described mechanisms leading to the modulation of vascular cell function and blood vessel tone and remodeling (2). The known mechanisms that underlie the preservation of vascular function include E2 inducing a favorable lipid profile, serving as an anti-oxidant, inhibiting the synthesis of prothrombotic proteins, and stimulating nitric oxide generation. However, the cell-based actions of E2, which lead to the preservation of the structure and function of important vascular cells, such as endothelial cells (EC), are not well understood. These actions are important because it has recently been appreciated that endothelial cell dysfunction underlies many acute and chronic vascular diseases (3).

Steroid hormones have traditionally been thought to act exclusively by binding to nuclear receptors, which then transactivate target genes (4). Recent evidence has additionally supported rapid, non-genomic actions of several steroids (5, 6). Moreover, the estrogen receptor (ER) has also been identified in the cell plasma membrane (7), serving largely undefined roles. Some effects of E2 appear to originate from the cell membrane, probably through binding plasma membrane ER that activates downstream signaling (8–11). However, the range of signal transduction pathways activated by the membrane ER has not been defined, and the potential roles of these pathways to mediate important E2 actions in target cells are largely unknown. Here, we investigated potential roles of the ER in the function of the EC. We report the novel observation that E2 induces the activation of the p38β member of the mitogen-activated protein kinase family, leading to the activation of the serine/threonine kinase, MAPKAP-2 kinase, and the phosphorylation of heat shock protein (HSP) 27. This signal transduction pathway in the EC substantially underlies the ability of E2 to preserve the actin cytoarchitecture during metabolic stress, rescue EC from hypoxia-induced apoptosis, and induce the migration of EC leading to tube formation. These findings implicate the membrane ER in the vascular cell protective actions of this steroid.

EXPERIMENTAL PROCEDURES

Materials—A p38β dominant-negative adenoviral vector was obtained from Jiahuai Han (Scripps), a dominant-negative MAPKAP-2 kinase expression plasmid was from Matthias Gaestel (Max Delbuck Center for Molecular Medicine, Berlin, Germany), and a triple serine mutant, non-phosphorylatable HSP27 expression vector was a gift from Lee Weber (University of Nevada).

Vascular Endothelial Cultures—Bovine aortic EC cultures were prepared as described previously (12) and plated as primary cultures in phenol red-free medium and in serum stripped with charcoal to remove steroids.

p38 and MAPKAP-2 Kinase Studies—EC were synchronized in the absence of serum for 24 h. For p38 or MAPKAP-2 kinase activity assays, the cells were incubated with E2 (10 nM) for 10 min based on preliminary time course studies. The cells were lysed, and the lysate was immunoprecipitated with protein A-Sepharose conjugated to antiserum for p38 or MAPKAP-2 kinase. Immunoprecipitated kinases were washed and then added to the proteins ATF-2 (for p38) or HSP27 (for MAPKAP) for *in vitro* kinase assays, similarly as described previously (12). This procedure was followed by SDS-polyacrylamide gel electrophoresis separation and autoradiography/laser densitometry.

Transient Transfections—BAEC (passages 4–5) were grown to 40–50% confluence and then transiently transfected with 1.5–10 μg of fusion plasmids depending upon the plate size and the amount of cells. Plasmids for transfection included dominant-negative MAPKAP-2 kinase (MK2-R76K) (13), control plasmids pKS$^+$ and pcDNA3, or a phosphorylation mutant HSP27 (pSV2711–3G) (14). Cells were incubated with LipofectAMINE liposome-DNA complexes at 37 °C for 5 h followed by overnight recovery in Dulbecco's modified Eagle's medium contain-

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[1] The abbreviations used are: E2, estradiol; EC, endothelial cell(s); MAPKAP, mitogen-activated protein kinase activated protein; ER, estrogen receptor; HSP, heat shock protein; CCCP, carbonyl cyanide *p*-chlorophenylhydrazone; BSA, bovine serum albumin; BAEC, bovine aortic endothelial cell; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

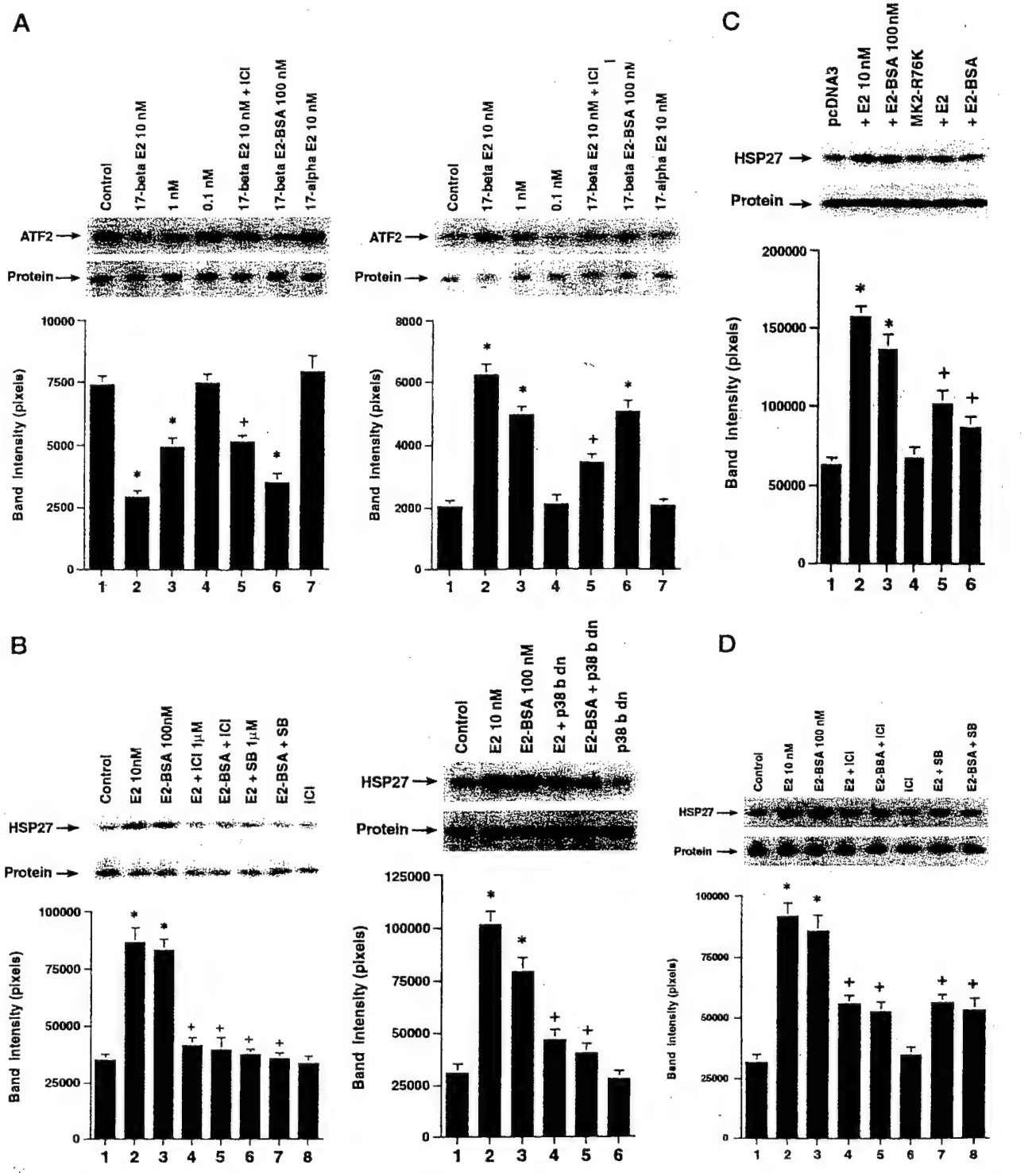

FIG. 1. *A*, 17-β-E2 or E2-BSA inhibits p38α but stimulates p38β activity in EC. EC were incubated with steroid for 10 min, and p38α (*left*) and p38β (*right*) were immunoprecipitated from the lysed cells and then used for *in vitro* assay of kinase activities directed against the ATF-2 substrate protein. Equal amounts of p38 protein are shown by the Western blot below the kinase activity. *B*, E2 or E2-BSA activates MAPKAP-2 kinase activity via p38β. SB203580 (*SB*) or infection of EC with a p38β dominant-negative (*dn*) adenoviral vector inhibited kinase activity. The *bar graphs* represent three experiments combined. *, $p < 0.05$ by analysis of variance plus Schefe's test for control *versus* condition; +, $p < 0.05$ for 17-β-E2 *versus* steroid plus ICI 182780 (*ICI*); (*left*) and 0.05 for 17-β-E2 *versus* 17-β-E2 plus SB203580 or dominant-negative p38β (p38TY) (*right*). E2 or E2-BSA stimulates the phosphorylation of endogenous HSP27 via p38 (*C*) and MAPKAP-2 (*D*) kinase. EC were either labeled with [^{32}P]orthophosphoric acid and then exposed to SB203580 or first transfected with a dominant-negative MAPKAP-2 kinase (*MK2-R76K*) followed by E2. *, $p < 0.05$ by analysis of variance plus Schefe's test for control *versus* condition; +, $p < 0.05$ for 17-β-E2 *versus* 17-β-E2 plus SB203580 or dominant-negative MAPKAP-2 kinase.

ing 10% fetal bovine serum. The cells were synchronized for 24 h in serum-free Dulbecco's modified Eagle's medium and then experimentally treated. For the p38β dominant-negative, a recombinant adenovirus expressing this construct (p38TY) (15) was added to subconfluent EC in Dulbecco's modified Eagle's medium with 2% serum, and infection occurred at a multiplicity of 50–100 particles/cell for 12 h. The cells were then cultured in serum-free medium for 36 h before experimentation.

Cytoskeletal Actin—Non-transfected or transfected EC were grown to confluence on poly-D-lysine-coated glass coverslips and then exposed to

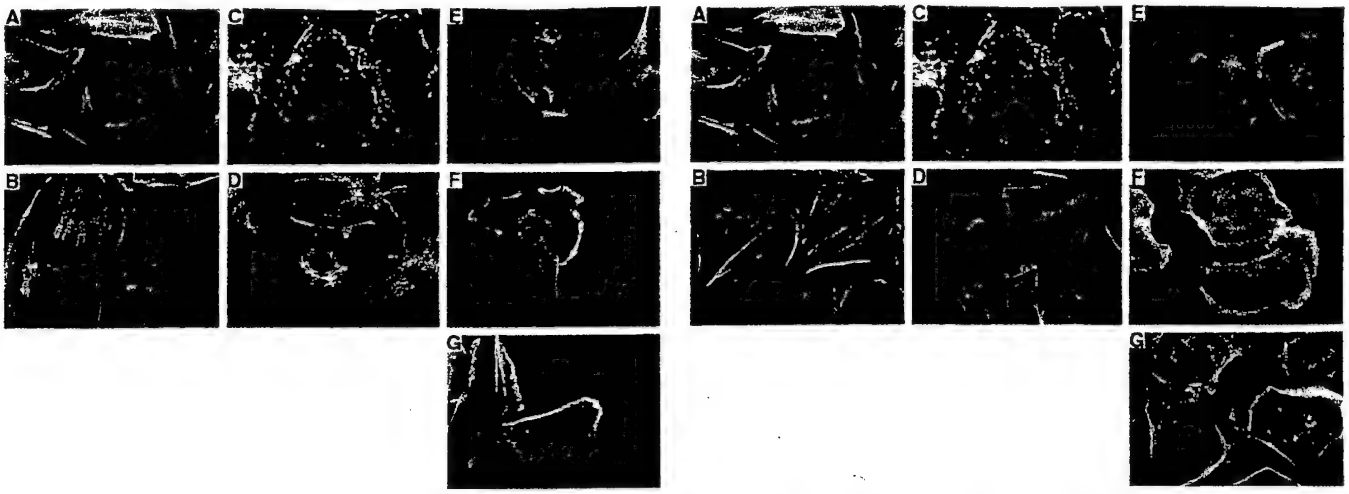

FIG. 2. **Metabolic stress disrupts the actin cytoskeleton in EC, whereas E2 (*left*) or E2-BSA (*right*) prevents this disruption via p38, MAPKAP-2, and phospho-HSP27-related mechanism.** *A* is control EC incubated in Dulbecco's modified Eagle's medium; *B* is with 10 nM E2; *C* is EC in 40 mg/dl glucose and exposed to 20 μM CCCP for 2 h; *D* is EC exposed to CCCP plus E2 (10 nM); *E* is CCCP plus E2 plus SB203580; *F* is CCCP plus E2 in EC transfected to express a dominant-negative MAPKAP-2. *G* is CCCP plus E2 in cells transfected to express a triple Ser-Ala mutant HSP27. *Right side* of figure is similar to *left side* except for using E2-BSA (100 nm) instead of E2. The representative experiment shown here was repeated twice.

20 μM CCCP, an uncoupler of oxidative phosphorylation in the presence of 40 ng/ml glucose for 2 h. Some cells were exposed to concomitant E2 (10 nM) or E2-BSA (100 nM). The cells were permeabilized, washed, and then stained with fluorescent-labeled phalloiden (Molecular Probes). Actin distribution was examined under a Nikon epifluorescent microscope.

Endothelial Cell Migration Assay—Non-transfected or transfected EC were grown to confluence on 6-well plates in Dulbecco's modified Eagle's medium with 10% serum. The cells were synchronized for 24 h in the absence of serum, and a wound was created by scraping the monolayer with a single-edge razor blade. Selected reagents were added to the wounded BAEC for 24 h at 37 °C. The cells were then fixed in 3.7% formaldehyde and assessed for migration. BAEC migration was measured using an image analyzer system composed of an inverted microscope and a 20–24-inch digitizing board (Jandel Scientific, Corte Madera, CA) attached to an IBM computer. The Sigma Scan program (Jandel) was used for analysis of measurements of the distance traveled by the cells within the calibrated area adjacent to the wound. Five measurements/well were taken, and the results from three separate experiments contributed to the bar graph (see Fig. 4).

Apoptosis Studies—Non-transfected or transfected EC were placed into an anaerobic chamber for 24 h (Gas Pack System, Becton-Dickinson), which was purged with 95% N_2, 5% CO_2 and sealed with an oxygen-consuming palladium catalyst. This created hypoxic conditions of 35 mm Hg PO_2. Some cells were exposed to 10 nM E2 or 100 nM E2-BSA. The control cells were subjected to normoxia (atmospheric air/5% CO_2, 150 mm Hg PO_2) in the absence of E2. Apoptosis was assessed by TUNEL staining, and the cells undergoing programmed cell death were counted in five separate fields/experiment. Data from three experiments constitute the bar graph (see Fig. 3).

Tube Formation—Human dermal microvascular EC were plated on growth factor-reduced Matrigel in the presence or absence of E2 and maintained for 6 h at 37 °C. The cells were fixed at 6 h (maximum tube formation), stained with Diff-Quik, and photographed and assessed at ×10 magnification using phase microscopy. Five random fields/condition were examined, the number of cords/tubes was counted in each, and the mean values were determined. The experiment was repeated twice additionally.

RESULTS

E2 Induces Rapid Activation of p38β, MAPKAP-2, and the Phosphorylation of HSP27—Endothelial cells have been previously demonstrated to contain ER (16). We first determined that in the primary cultures of bovine aortic endothelial cells, E2 stimulated the activity of the mitogen-activated protein kinase family member, p38β (Fig. 1*A*, *right*) while inhibiting p38α activity (Fig. 1*A*, *left*) maximally at 10-min incubation. This finding indicates a unique differential regulation of the isoforms of this kinase. E2 effects were significant at 1 nM and were substantially reversed by the specific ER antagonist, ICI 182,780. No effects were seen with 10 nM of the relatively inactive stereoisomer 17-α-E2 or with testosterone, but a cell-impermeable membrane ER ligand, E2-BSA, affected the activity of the two isoforms of p38 comparable to E2. E2-BSA has been shown in several studies to neither enter the cell nor bind and/or activate the nuclear ER (10, 17, 18). These data implicate a membrane ER in the modulation of signaling, mainly based upon the rapid and specific effects of E2 but also based upon the lack of precedence for any nuclear receptor to rapidly signal, as well as the comparable effects of E2-BSA and E2.

Little is known in general about the cell biologic importance of p38β except that it might participate in cardiac hypertrophy (19). However, a known immediate downstream target of p38 is the serine/threonine kinase MAPKAP-2 (20). We found that E2 or E2-BSA was each capable of stimulating the activity of this kinase in EC directed against a substrate protein, exogenous heat shock protein 27 (Fig. 1*B*). The stimulation of MAPKAP-2 kinase activity was reversed by the soluble and specific inhibitor of p38 activity, SB203580 (21), or by infecting the EC with an adenoviral vector expressing a dominant-negative mutant p38β protein (15). The results indicate that the β-isoform of p38 mediates the actions of E2 to phosphorylate exogenous HSP27.

We then sought to link this pathway to endogenous HSP27 phosphorylation, a known substrate for MAPKAP-2 kinase. E2 or E2-BSA stimulated the phosphorylation of endogenous HSP27, and this was reversed by SB203580 (Fig. 1*C*). Transiently transfecting and expressing a dominant-negative MAPKAP-2 protein MK2-R76K (13) also abrogated the phosphorylation of HSP27 in response to the steroid (Fig. 1*D*). This process identified a novel and rapid signal pathway for estrogen, potentially contributing to the steroid cellular actions.

E2 Signals to the Preservation of the Actin Cytoskeleton—HSP27 is phosphorylated by MAPKAP-2 at three serine residues at positions 15, 78, and 82 (22). HSP27 acts as a capping protein for the barbed ends of actin in the unphosphorylated state (23) but is believed to play a role as a phosphoprotein in stabilizing F-actin and allowing the polymerization of this myofibril (24). In quiescent EC, a predominantly cortical pattern of F-actin localization was observed (Fig. 2*A*, *left*). Upon exposure to E2, the actin filaments were localized to stress fibers and focal adhesions (Fig. 2*B*, *left*). We exposed the EC to an uncoupler of oxidative phosphorylation, CCCP (20 μM), in the pres-

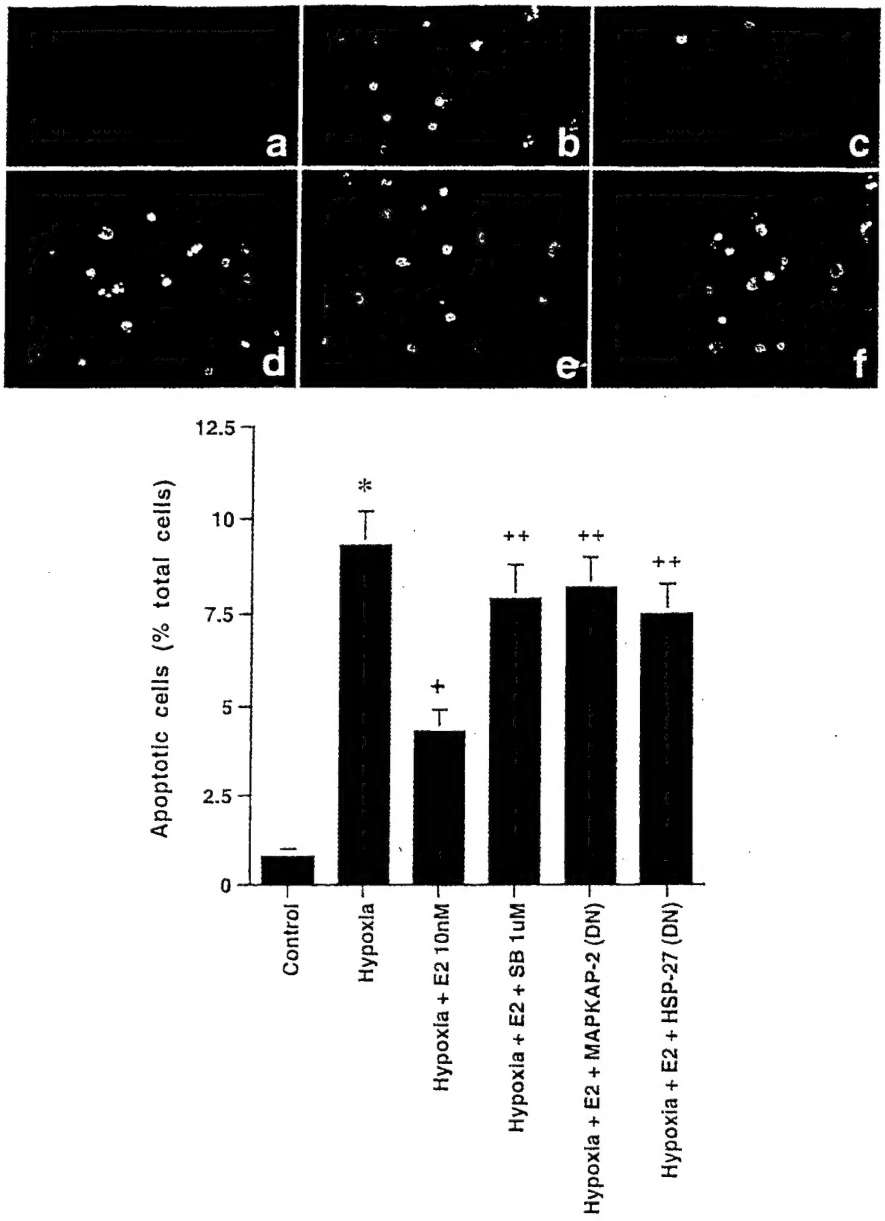

FIG. 3. **Hypoxia induces apoptosis of EC, prevented by E2 signaling.** EC exposed to 1% O_2 underwent cell death (*b*) reversed by E2 (*c*). The effects of E2 were prevented by inhibiting p38 activation (*d*), MAPKAP-2 activation (*e*), or by the expression of the dominant-negative HSP27 (*f*). *a* is EC exposed to room air. *Bar graph* is three experiments combined. *, $p < 0.05$ by analysis of variance plus Schefe's test for control *versus* hypoxia; +, $p < 0.05$ for hypoxia *versus* 17-β-E2 plus hypoxia; ++, $p < 0.05$ for hypoxia plus E2 *versus* 17-β-E2 plus hypoxia plus SB203580 (*SB*), dominant-negative (*DN*) MAPKAP-2, or serine mutant HSP27.

ence of low glucose; this metabolic stress simulates an ischemic insult (25). The stress led to the distortion of the actin cytoarchitecture, the severing of actin, and a severely disrupted stress fiber and focal adhesion formation (Fig. 2*C, left*). Incubation of the cells with E2 strongly preserved the cell membrane integrity and stress fiber/focal adhesion localization of the actin (Fig. 2*D, left*). However, treatment of the cells with SB203580 or expression of the dominant-negative MAPKAP-2 protein substantially prevented the effects of E2 (Fig. 2, *A*, *E*, and *F, left*). Similarly, the expression of a triple serine mutant HSP27 that is unable to be phosphorylated reversed the ability of E2 to preserve cytoskeletal integrity in the stressed EC (Fig. 2*G, left*). Similar results were seen when the EC were exposed to E2-BSA (Fig. 2, *right*). The stable and localized expression of actin to stress fibers is necessary to preserve cell morphology and function and prevent EC dysfunction, which is strongly implicated in the pathogenesis of vascular disease (3). This expression identifies a potentially important cell biologic effect of E2.

E2 Prevents EC Cell Death Resulting from Hypoxia—We then explored other cell biologic effects of E2 in this context. EC become dysfunctional or undergo cell death after acute/chronic ischemia or hypoxia. We subjected the EC to 24 h of hypoxia in a chamber containing 1% O_2. This resulted in substantial apoptotic cell death as shown by TUNEL staining (Fig. 3, *b versus* control *a*). E2 was capable of rescuing the cells, preventing 64% of the hypoxia-induced apoptosis (Fig. 3*c*), but this was substantially reversed by inhibiting p38 or MAPKAP-2 kinase activation (Fig. 3, *d* and *e*) or by expressing the non-phosphorylatable HSP27 (Fig. 3*f*). Similar protection was afforded by the E2-BSA ligand (data not shown). Thus, this pathway serves an important function for cell survival.

E2 Stimulates EC Migration and Tube Formation—The migration of vascular cells is critical to the process of blood vessel remodeling. For instance, the migration of a subpopulation of endothelial cells is an important step in the process of angiogenesis. We determined whether E2 could promote EC migration via a signaling-related mechanism. E2 clearly promoted the migration of EC across a wound barrier (Fig. 4, *A versus B* and *bar graph*) and was not capable of stimulating EC proliferation in 24 h, which supported migration as the mechanism. The effect of E2 was prevented by 85% from the incubation of cells with SB203580 (Fig. 4*C*) and was substantially reversed by the expression of MAPKAP-2 dominant-negative protein

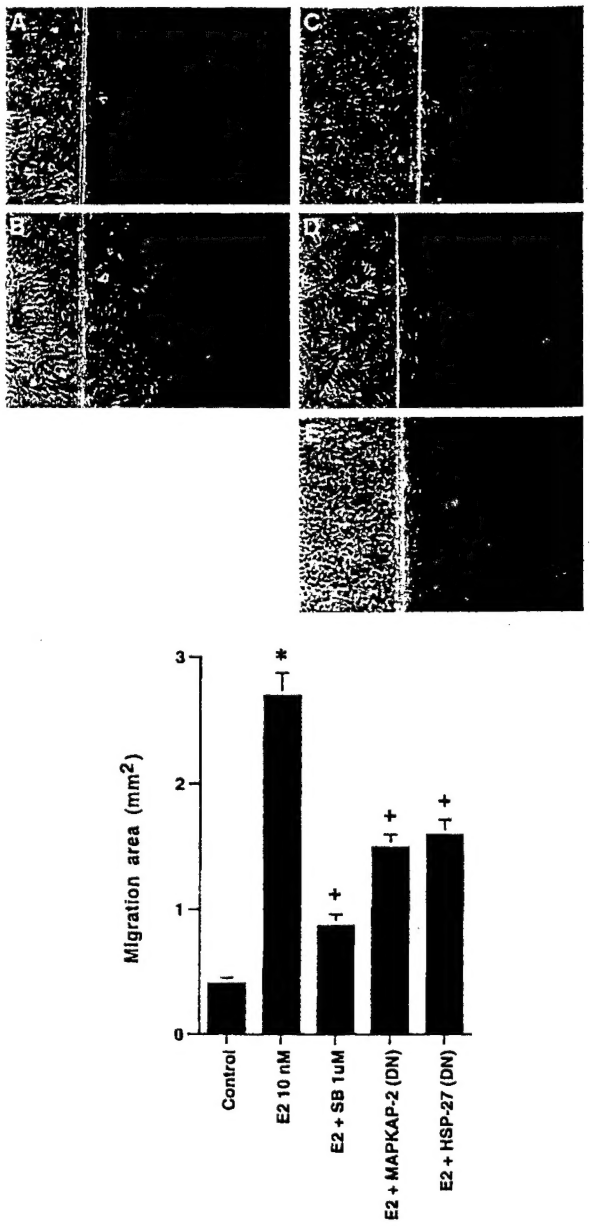

FIG. 4. **E2 induces the migration of EC that is prevented by the inhibition of the activity of p38 and MAPKAP-2 kinase and that is expressing a non-phosphorylatable HSP27 protein.** *Top*, non-transfected or transfected BAEC were cultured on agar and were wounded by a razor blade. Migration across the wound was determined 24 h later. *A* is control; *B* is EC incubated with E2 (10 nm); *C* is E2 plus SB203580; *D* is E2 incubation of EC expressing a dominant-negative MAPKAP-2 protein; *E* is in EC expressing a triple serine mutant HSP27 plus E2. The *bar graph* (*bottom*) is from three experiments. *SB*, SB203580; *DN*, dominant negative.

(Fig. 4*D*) or the expression of the phosphorylation mutant HSP27 (Fig. 4*E*). These results support the idea that signal transduction from the membrane ER is necessary for E2 to promote this important step in angiogenesis. E2-BSA again simulated these actions of E2.

Previous work suggests that E2 may stimulate angiogenesis (26), as exemplified by the sex steroid-entrained growth and regression of blood vessels in the uterus during the course of the menstrual cycle. Classical angiogenic growth factors, such as vascular endothelial growth factor, signal to the various steps of new blood vessel formation (12). Capillary tube formation *in vitro* has been used as a model of the early organization of new blood vessels (26). To explore the direct effects of E2, we plated human dermal microvascular EC onto growth factor-deficient Matrigel, a protein substratum mixture that supports the organization of EC into tubes. All cells were incubated in the absence of exogenous growth factors, serum, or phenol red medium. EC incubated with E2 or E2-BSA developed into branching, cordlike primitive capillaries to a much greater extent than the media-incubated control cells during a 6-h period (Fig. 5, *a versus b* or *c*). This effect was again substantially reversed by SB203580 (Fig. 5, *e* and *f*), whereas SB203580 alone had little effect (Fig. 5*d*). Expression of MK2-R76K (Fig. 5*g*) substantially reduced E2 or E2-BSA-induced capillary formation (Fig. 5, *h* and *i versus b* and *c*). Similar results were seen with the expression of the triple serine mutant HSP27 (Fig. 5, *k* and *l versus b* and *c*). These results suggest that the activation of this signal transduction pathway underlies the ability of E2 to stimulate the early stages of capillary tube formation.

DISCUSSION

Steroids traditionally are thought to exclusively act in the cell as nuclear transcription factors, modulating target genes through complex interactions of steroid receptors, co-activators or co-repressor proteins, histone acetylase, and proteins comprising the basal transcriptional machinery (27). However, a functional plasma membrane ER was identified more than 20 years ago (7), and additional evidence has supported its existence and suggested a potential role in the biology of estrogen action (8–10). As a plasma cell membrane receptor, ER might be expected to modulate signal transduction, and several pathways have recently been identified as being activated by E2 (9–11, 28). Here, we report novel findings that E2 can activate a signal pathway that leads to the preservation of function and form of vascular endothelial cells (Fig. 6). These results define several mechanisms by which E2 can protect against EC dysfunction and resulting vascular disease (3).

We first found that E2 or E2-BSA activated the p38β isoform but inhibited p38α at 10 min. A specific ER antagonist reversed these effects. We, therefore, propose that a membrane ER rapidly regulates these kinases. Furthermore, mitogen-activated protein kinases localize to the plasma membrane where they are activated by upstream signal transduction pathways, which originate at this location (29). Activation of the p38β member of the mitogen-activated protein kinase family has not been reported for any steroid, and the differential modulation of p38α and p38β isoforms by any substance has not been previously reported. The p38 family consists of four isoforms (α, β, δ, γ), and the precise cellular roles of each are incompletely understood. p38α has been shown to participate in the induction of apoptosis (30, 31), whereas p38β is known to participate in cardiac hypertrophy (19) and mediates the transcriptional regulation of Bcl-2 and anti-apoptosis (32). Interestingly, we found that estrogen inhibits p38α in the EC. Taken together, modulation of these two isoforms of p38 may contribute to anti-apoptosis in the EC (see below).

We determined that E2 or E2-BSA comparably activated p38β and the downstream signaling and endothelial cell biology. Several laboratories (17, 33), in addition to our own (9, 10), have provided evidence that E2-BSA does not enter the cell to bind the nuclear ER or dissociate into E2 and BSA components as implicated in a single publication (34). Also, considering 1) the rapidity of E2 (or E2-BSA) effects, 2) that a specific ER antagonist can prevent the signaling by the steroid compounds actions, and 3) the fact that there is no precedent for nuclear receptors to rapidly signal, we therefore believe that these non-genomic effects of E2 are mediated at the membrane ER.

The activation of p38 by E2 then led to the activation of the MAPKAP-2 kinase, a member of a serine/threonine family of related kinases that is stimulated by a variety of cell stressors

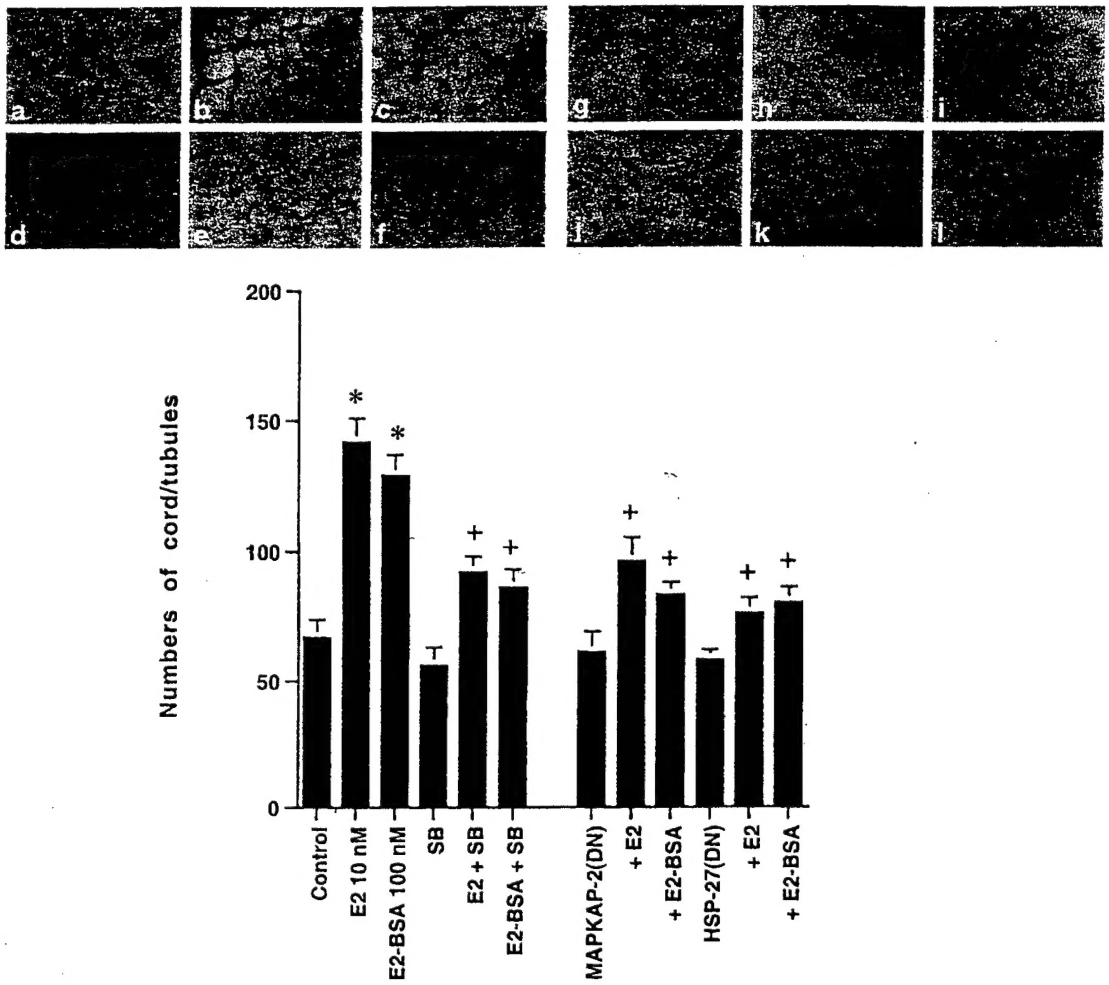

FIG. 5. **Capillary tube formation occurs in response to E2 signaling.** Human microvascular EC minimally developed into capillaries 6 h after plating on GFP-Matrigel (*a*). E2 or E2-BSA increased the number of tubes formed (*b* and *c*) that were reversed by the inhibition of p38 with SB203580 (*SB*) (*e* and *f*). Expression of dominant-negative MAPKAP-2 (*DN*) (*g*) inhibited capillary formation by E2 or E2-BSA (*h* and *i versus b* and *c*) as did the expression of a mutant HSP27 in the setting of the steroids (*k* and *l*). *Bar graph* data are means ± S.E. of the capillary tube number from three experiments.

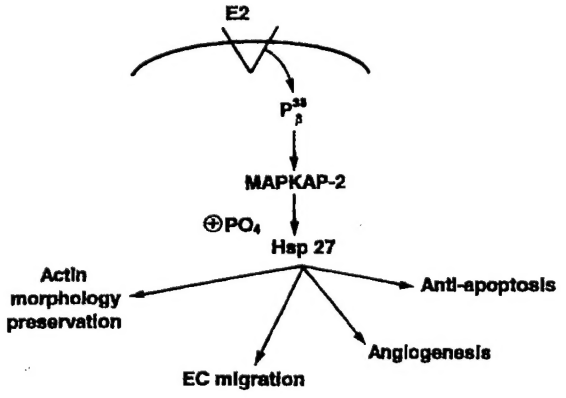

FIG. 6. **E2 signaling to EC-related functions through p38 mitogen-activated protein kinase, MAPKAP-2 kinase, and HSP27.**

and growth factors. MAPKAP-2 kinase mediates the direct phosphorylation of transcription factors (cAMP-response element-binding protein, serum response factor) (35, 36) or acts indirectly through downstream kinases, such as MSK-1 (37). In our studies, E2 induced the phosphorylation of endogenous HSP27, and this required MAPKAP-2 kinase because the expression of a dominant-negative MAPKAP-2 significantly prevented HSP27 phosphorylation. Expression of this dominant-negative MAPKAP-2 construct has been previously shown to prevent the activation of this kinase (13), and we confirmed this in response to E2 or E2-BSA (data not shown).

What is the importance of the ability of estrogen to trigger this signaling pathway? The integrity of F-actin is necessary for the physiological functions of EC, such as creating a selective vascular permeability barrier to proteins (38, 39). Disruption of the actin cytoskeleton occurs after ischemia or other stresses and results in tissue edema formation or vascular thrombosis (40, 41). This disruption is because of ATP depletion during ischemic stress, a metabolic condition that we have simulated here with low glucose and an inhibitor of oxidative phosphorylation (25). Upon subjecting the EC to this stress, the F-actin integrity and localization were markedly disrupted. E2 or E2-BSA was capable of preventing this disruption through signaling to HSP27 phosphorylation. In this respect, EC survival is also related to our demonstrated effects of E2. HSP27 is a known survival factor for a variety of cells (42, 43) perhaps through helping to maintain cytoskeletal integrity, thus preventing apoptosis in response to several stresses in various cell types (44, 45).

ATP depletion results in cellular calcium overload, which can activate gelsolin, an actin-severing protein (46). It is unknown but possible that phosphorylated HSP27 can prevent gelsolin or related protein activation in this setting. Phosphorylated HSP27 also promotes EC migration that is (47) in part related to the ability of the phosphoprotein to allow F-actin polymeri-

zation (23), which is critical for the extension of cell processes, such as lamellopodia (48). Cell migration is dependent on a complex series of events that include active signaling from components of the focal adhesion complex, such as Src family kinases and focal adhesion kinase (49). It has recently been shown that E2 can activate Src in MCF-7 cells (10, 50). Proximal signaling to the activation of the focal adhesion complex, possible activation of the small GTPases (Rac and Rho), and the activation of the p38-mediated signaling pathway described here may be linked or may occur in parallel in response to E2.

Cell migration is one important step in the process of angiogenesis. An excellent example of hormone-entrained neovascularization/regression of blood vessels occurs in the uterus during the menstrual cycle. Angiogenesis may result in part from the ability of E2 to stimulate local vascular endothelial growth factor production (51) and subsequent neovascularization. Here, we show a direct effect of E2 in stimulating primitive capillary tube formation, at least in part, through signaling to HSP27 posttranslational modification.

The actions of E2 to signal to cell biology through the membrane ER do not preclude important effects of the nuclear ER to transcribe genes that are critical to maintain EC function. For instance, E2 can transcriptionally activate the HSP27 gene through an Sp1-related mechanism (52). This indicates an important synergism where the nuclear ER promotes HSP27 protein synthesis, and the membrane ER probably triggers the rapid, function-regulating phosphorylation of the protein. In this way, acute and chronic modulation of HSP27 can be achieved through the potentially coordinated actions of distinct and compartmentalized pools of ER.

In total, these effects of E2 remarkably preserve EC form and function in the setting of several relevant *in vitro* stresses. This interaction may unfortunately extend to breast cancer where HSP27 expression and function correlate with the survival and invasiveness of tumors (53, 54), both of which are promoted by E2. This may be related to the dependence of the tumor vasculature on the survival and preservation of EC function that is induced by E2 in an HSP27-dependent fashion as shown here.

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